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SUPERINFECTION IN EXPERIMENTAL SYPHILIS FOLLOWING THE ADMINISTRATION OF SUBCURATIVE DOSES OF ARSPHENAMINE OR NEOARSPHENAMINE.*

By WADE H. BROWN, M.D., and LOUISE PEARCE, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

Plates 73 and 74.

(Received for publication, February 21, 1921.)

It is generally held that a person infected with the virus of syphilis becomes practically immune to a second infection—that, with the development of the initial lesion, a condition becomes established which makes it difficult or impossible to superimpose a second infection upon the one already present and that this refractory state is maintained as long as an infection exists. As far as they have been tested, human and animal experiences are in essential agreement upon these points.

In the absence of any evidence to the contrary, it has been assumed that the principles contained in this conception of syphilitic immunity apply to treated as well as to untreated cases of infection and many syphilographers have regarded the appearance of fresh lesions of the chancre type, under circumstances which would indicate a new infection, as the most conclusive evidence of the cure of the previous infection. There are some, however, who have questioned the validity of so called reinfections, and others who have objected to their interpretation as evidence of cure. Granting that the class of cases referred to represents instances of true, second infection, an alternative interpretation of such occurrences has recently been presented by Jacobi (1) in which it is suggested that the reaction to a second inoculation may be viewed more as an expression of an existing state of immunity than as evidence of the presence or absence of infection.

* The results of the experiments reported in this paper were demonstrated at the All-America Conference on Venereal Diseases, Washington, D. C., December 6 to 11, 1920.

The situation which exists with reference to the immunity of infected individuals, the possibilities of superinfection or reinfection, and hence the interpretation to be placed upon the appearance of manifestations of disease which give every evidence of being due to a new infection are both complicated and obscure. Until the introduction of modern methods of spirocheticidal therapy, instances of so called reinfection were comparatively rare, and interest in the subject was largely a theoretical one. Within the past few years, however, the literature has contained numerous reports of reinfection following treatment with arsphenamine or neoarsphenamine, and the interpretation to be placed upon these occurrences has become a matter of immediate practical importance. Where the evidence of a new infection seemed sufficient, the general tendency has been to accept such infections as proof of a cure, and this would appear to be the logical interpretation unless it can be shown that under circumstances such as have existed in these cases, superinfection becomes possible. An element of uncertainty is introduced by the treatment employed, since practically nothing is known concerning the influence of such drugs as arsphenamine and neoarsphenamine upon syphilitic immunity.

The point at issue, therefore, is not so much a question of the immunity conferred by syphilitic infection as it is the effect which given therapeutic agents may have upon the resistance of infected individuals, whether native or acquired, and upon any spirochetes which may survive their action. Presented in this form, the problem of reinfection may be approached experimentally as one of the influence of drug action upon immunity and infection, and experiments have been carried out in animals for the purpose of determining the effect of subcurative doses of arsphenamine and of neoarsphenamine upon the resistance of infected animals to reinoculation. These experiments were divided into two groups according to the stage or progress of the infection, and the work to be reported in this paper deals with the results of treatment and reinoculation of animals with early but marked primary lesions.

EXPERIMENTAL.

The experiments to be reported consisted of the infection and treatment of two sets of rabbits—one with arsphenamine and the other with neoarsphenamine—after which they were reinoculated for the

purpose of determining their susceptibility to a new infection as indicated by the production of lesions at the site of inoculation. The results of the experiments were controlled in four ways, (1) as to the effects of the treatment employed, (2) as to the immunity developed by the infection, (3) as to the relative susceptibility of normal animals as indicated by their reaction to the virus used in the reinoculation experiments, and (4) by the use of a virus of essentially the same virulence as that causing the existing infection.

All animals were kept under observation for a minimum of 8 weeks after treatment while the therapeutic controls were held for 4 months as a means of affording a more accurate estimation of the status of the infection existing after treatment.

Infection of the Animals Used.—The animals used were inoculated in both testicles with 0.2 cc. of an emulsion containing one to three spirochetes to the microscopic field (Nichols strain). The object in view was the production of an intense infection which would confer a high degree of protection against reinoculation within a short period of time. The incubation period of the testicular lesions averaged about 10 days and the lesions developed very rapidly; at the end of 18 days the testicles were markedly enlarged and indurated, and in some instances there was an edema of the scrotum, indicating that the lesions were approaching the height of their first cycle of development. The animals were then divided into three groups according to the degree of development of the testicular lesions: Five animals with the most advanced infections were placed in the group to be treated and reinoculated; five others with the least advanced lesions were set aside as infected controls to be reinoculated at the same time as the treated animals; while a third group, consisting of animals showing various degrees of testicular involvement, were treated as therapeutic controls for the reinoculated animals of the first group.

Treatment.—Treatment was carried out 18 days after inoculation by the intravenous administration of a single dose of arsphenamine or neoarsphenamine. The products used were of the original German manufacture, being from Lots A 25819 and A 25884 respectively. The arsphenamine was neutralized by the addition of the theoretical amount of N sodium hydroxide to form the disodium salt, and both substances were administered in a 0.2 per cent solution.

The drugs were employed in the equivalent amounts as stated by the manufacturer. The doses used were 6 mg. of arsphenamine per kilo of body weight and 9 mg. of neoarsphenamine. The selection of these doses was based upon known values of therapeutic action for arsphenamine rather than neoarsphenamine and represented an attempt to use a dose of this drug which in the average animal of the group would yield a therapeutic effect of a definite character; namely, a regression of lesions approximating complete resolution with freedom from recurrence for 4 to 6 weeks followed by clinical relapse within a period of not more than 2 to 3 months. The ability to gauge these effects correctly was considered to be one of the two most essential features of the experiments, the other being reinoculation.

Twelve rabbits were treated with each of the drugs in the manner described; five of them were subsequently reinoculated and the seven others held as therapeutic controls.

Reinoculation.—Reinoculation of treated and untreated animals was carried out on the 24th day of the infection (5 days after treatment). In order that there might be as little difference as possible in the virulence of the organisms used for reinoculation and those producing the original infection, the virus used was obtained from an animal of the same series as those to be reinoculated. This was, in a sense, a measure of control. Each animal received an intracutaneous injection of 0.2 cc. of an emulsion containing one to three spirochetes to the microscopic field at two widely separated points, the ventral surface of the sheath and the base of the right ear in the region of the posterior marginal vein (see illustrations). It will be noted that one of these points was in close proximity to the original lesions and the other as far removed as was practicable. This choice of sites for reinoculation was determined in part by the susceptibility of skin areas and in part was used as a means of checking the extension of the immunity in control animals.

Reinoculations were timed both with reference to the possible retention of drug in a biologically active state and with reference to the progress of drug effects, the intention being to reinoculate as early as possible so as to give the best opportunity for the development of lesions before recurrence of the original lesions took place.

Methods of Control.—The results of the experiments outlined were controlled in four ways:

1. Therapeutic Controls.—In order to avoid any confusion which might arise from an attempt to interpret therapeutic effects obtained in animals which had been reinoculated, seven rabbits from each of the treated groups were held under observation for the purpose of determining as nearly as possible the effect which had been produced upon the original infection by the treatment employed.

2. Infected Controls.—Five infected rabbits of the same series as those used for treatment were reinoculated in the manner described with the same material as that used for reinoculation of the treated animals and for the inoculation of the normal controls.

3. Normal Controls.—The relative susceptibility of normal animals to the virus used for reinoculation was controlled by the inoculation of three normal rabbits carried out in the same manner as that of treated animals. These will be referred to as normal controls.

4. Virus Control.—As a means of insuring equality in the virulence of the spirochetes originally introduced and those used for the second inoculation, the reinoculations were made with material taken from a testicle of an animal of the same series as those to be reinoculated.

Effects of Treatment.

A consideration of the results obtained from the experiments outlined above should logically begin with the effects of the treatment employed. Following administration of the drugs, the testicular lesions began to regress, resolution proceeding somewhat more rapidly in the animals treated with neoarsphenamine than in those with arsphenamine. In some animals, the lesions disappeared completely by the end of 7 to 14 days, at which time the effect of the drug upon existing lesions practically ceased.

Of the twelve animals treated with neoarsphenamine, the testicular lesions were completely resolved in seven, four showed definite residual lesions in the form of diffuse thickenings or of circumscribed nodules, and in one the result was uncertain. In contrast to this, complete resolution occurred in only four of the animals treated with arsphenamine—a difference which is quite characteristic of the action of the two drugs in inducing resolution or healing of lesions in the experimental animal.

The first evidences of relapse among the therapeutic controls were noted between 14 and 17 days after treatment. Four of the seven animals treated with arsphenamine showed reinduration and gradual increase in the size of residual lesions or the development of new foci of infection by the end of the 3rd week, and the infection progressed at a normal rate. A fifth animal relapsed 45 days after treatment, while the condition in the two others was not clear. From the 3rd week onward, there were brief periods during which the testicles of these two animals appeared to enlarge somewhat, there were ill defined areas of thickening, and even a few tiny nodules in the testicles or tunics, all of which were suggestive of relapse, but none of these conditions developed into affections which exhibited the characteristic clinical appearance of syphilitic lesions. At the end of 3 months, test inoculations were made from popliteal lymph nodes of both animals with positive results, and one of them developed a testicular lesion shortly afterwards.

Clinical relapse among the animals treated with neoarsphenamine was more delayed. Three animals of the control group showed minor changes suggestive of relapse as early as 14 days after treatment, but outspoken lesions did not develop until towards the end of the 3rd month. Relapse occurred in the four others between the 24th and 39th days, but in two of these the growth of the lesions was again rather slow and irregular for from 4 to 6 weeks.

Infection was, therefore, shown to be present in all the therapeutic controls of both series. In other words, none of the animals were cured by the treatment received, and similar but less marked effects were produced by the treatment given the animals which were used for reinoculation.

Results from Reinoculation of Infected Controls.

The second point to be considered is the probable state of immunity which had developed in the treated and reinoculated animals. It was not possible to determine this with certainty, and in reality such a determination was not essential to the object of these experiments. However, as the best means of estimating the immunity in these rabbits and of controlling the results of reinoculation at the same time,

which was of more importance, animals with the most advanced infections were selected for treatment, while those showing the least progress of the infection were used as controls. In this way, the state of the infection existing at the time of reinoculation of the untreated animals was practically the same as that which had existed at the time of treatment of the other group.

The results from reinoculation of the infected controls may be given briefly. Within a few hours, all animals of the series showed a slight acute reaction at the site of inoculation which consisted first of an edema and then of a slight diffuse redness about the site of inoculation. This reaction subsided completely within 24 to 48 hours and in one animal was the only reaction observed. In the others, a diffuse or papular infiltration developed in the sheath and at the base of the ear. The lesions in the sheath reached their height in from 5 to 7 days and disappeared completely within 12 to 14 days. The papules measured from 3 to 6 mm. in cross-diameter; they were of a rose-pink color, firmly indurated, and of a slightly translucent appearance. The more diffuse lesions presented essentially the same characteristics. The ear lesions developed somewhat more slowly, and in three of the five animals were very slight, diffuse infiltrations lasting approximately 3 weeks. The majority of the lesions described were of the type of slight non-specific inflammatory processes, or they might be regarded as allergic reactions. It is possible that some of them were due to a slight but transient local infection, but no examination was made for spirochetes for fear that the trauma inflicted might induce regression.[1]

In two instances out of the ten inoculations (two injections in each animal), small, firmly indurated, and translucent papules developed at the base of the ear (Figs. 1 and 2). Clinically, the lesions presented the appearance of syphilitic granulomata. One of them disappeared within 3 weeks, and while the other never developed to more than 4 to 6 mm. in diameter, it persisted for 54 days.

These were the only instances in which reinoculation resulted in the production of skin lesions which gave evidence of being due to

[1] While trauma in some form appears to play a part in the distribution and even in the development of syphilitic lesions in the rabbit, scarification, cutting, or aspiration with a needle frequently causes them to regress.

infection, and they are reproduced in Figs. 1 and 2 for purposes of comparison with the lesions obtained by reinoculation of the treated animals. It is to be noted that both lesions occurred on the ear, while the foreskin was entirely negative, which is the reverse of the order of susceptibility of the two skin areas in normal rabbits.

Results from Reinoculation of Treated Animals and Normal Controls.

Reinoculation of treated animals gave results which were strikingly different from those of the infected controls. In a word, all except two of them developed perfectly typical chancres, examples of which are given in Figs. 3 to 12; there was marked lymphadenitis such as is associated with primary lesions, and spirochetes were present in abundance. Within 7 days, every animal of the group showed a characteristic syphilitic reaction at the site of inoculation, either in the form of an elevated papule or of a flattened area of infiltration. Of the twenty points inoculated, nineteen were positive by the 7th day, and a lesion appeared at the one remaining focus on the 11th day after inoculation. The incubation periods of these lesions coincided with those of the normal controls.

The growth of both the sheath and ear lesions in four of the ten animals, including two treated with arsphenamine (Figs. 3 to 7) and two with neoarsphenamine (Figs. 8 to 12), was extremely rapid and practically uninterrupted until they reached the stages of development shown in Figs. 3 to 12, and some of them progressed beyond the points shown. On the other hand, none of the three normal controls developed lesions which were at all comparable to the sheath and ear lesions of these four treated animals.

In another animal (arsphenamine), the lesion on the sheath grew somewhat irregularly, but within 4 weeks formed a characteristic ulcerated chancre measuring 1 cm. in diameter. Growth then ceased for a short time, but the lesion was considerably larger and increasing actively when the animal was discarded. The ear lesion was of the nature of a papule surrounded by a zone of diffuse infiltration. It developed to approximately 8 mm. in diameter during the first 4 weeks but had practically disappeared before the animal was discarded.

The lesions of a sixth animal (neoarsphenamine) grew more rapidly than those of any other for about 3 weeks, but development ceased

at this point. On the sheath, there was a lenticular lesion measuring 5 to 7 mm. in thickness at its center and spreading diffusely over an area more than 1 cm. in diameter. A similar but less elevated lesion developed at the base of the ear, and while there was some exfoliation over the center of both lesions, neither of them underwent ulceration. They gradually subsided and had practically disappeared at the end of the 2 month period of observation. It is noteworthy that this animal developed a slight periostitis of the nasal bones 39 days after treatment and later a lesion of the cornea. There was also a marked popliteal lymphadenitis such as is commonly associated with focal infections in the drainage area.

In two other animals of the arsphenamine group, the reaction during the first few weeks after reinoculation was comparatively slight. During the 4th week, however, both the ear and sheath lesions of one animal began to increase rapidly and developed into characteristic chancre-like lesions of approximately 1 cm. in cross-diameter. The ear lesion of the other animal was first a papule, then a diffuse infiltration, but a typical chancre measuring 8 mm. in diameter developed on the sheath.

The lesions produced in the two remaining animals of the treated and reinoculated series were comparatively slight and consisted of small papules or diffuse infiltrations. They were more pronounced and more enduring than any lesion of the infected controls but less than those of the normal controls. The therapeutic response in one of these animals, both of which were treated with neoarsphenamine, was apparently less than that in any other animal of the series. The original lesions were never completely resolved, and a clinical relapse was recognized 17 days after treatment.

A feature of especial interest in these experiments was the fact that relapse of the original lesions occurred in eight of the ten reinoculated animals at about the time the superinfection lesions became well established. In most instances, the second chancres overgrew (inhibited) the recurrent lesions, but in other animals, both sets of lesions developed together.

An excellent illustration of this phenomenon of the double infection is furnished by Figs. 6 and 7. Inspection of Fig. 6 will show that there is a diffuse enlargement of the right testicle and two large nodules are well outlined at the positions indicated by the arrows. There is also a small patch of infiltration in

the left scrotum. The nodules in the testicle existed from the time of treatment, and although they were considerably softened and diminished in size, they became reindurated and began to enlarge by the end of the 2nd week. The development attained within 5 weeks (the time represented by this photograph) would of itself preclude the possibility of a metastatic origin. The further development of the two sets of lesions is shown in Fig. 7.

The results of the treatment and reinoculation experiments as a whole may be stated as follows: Of the five animals treated with arsphenamine, and then reinoculated, the original lesions were completely resolved in only one instance and relapse occurred within 33 days in four of the five animals, including the one animal whose lesions had been resolved. The other animal of the group showed no definite increase in the testicular lesions during the period of observation, and the lesions which resulted from reinoculation were less marked than those of the other animals. By reinoculation, characteristic chancres were produced in all the animals of the group. Three of the five animals developed well marked lesions at the base of the ear as well as on the sheath, while the ear lesions were comparatively slight in the two others.

The results after treatment with neoarsphenamine were not so uniform. The testicular lesions were quickly resolved in two of the five animals. The testicles of a third animal were left slightly enlarged and diffusely thickened, but no further change occurred, either in the way of regression or progression of the lesions during the period of observation, and while well marked lesions were produced by reinoculation on both the ear and the sheath, they were of shorter duration than those of the other animals. Clinical relapse occurred in all other animals of the group at from 14 to 24 days after treatment. Characteristic chancres were obtained from the second inoculation in three of the five animals on both the sheath and ear; the other two gave only diffuse or papular infiltrations.

The three normal controls developed characteristic chancres on the sheath which measured from 1 to 1.5 cm. in diameter. One of them developed a large nodule in the subcutaneous tissues at the base of the ear in addition to a diffuse infiltration of the skin and the usual lymphadenitis. In the two others, there were small papules surrounded by a zone of diffuse infiltration, but none of them developed chancre-like lesions in this location.

DISCUSSION.

In attempting to give an interpretation of the experiments reported, the first point to be considered is that while one group of animals with well developed primary lesions of the testicles proved to be extremely refractory to a second cutaneous inoculation with a virus of equivalent virulence, a second group of animals from the same series, after treatment with arsphenamine or neoarsphenamine, was highly susceptible and with two exceptions reacted to the second inoculation with the development of characteristic manifestations of a primary infection. In fact, if susceptibility may be gauged by the reaction at the site of inoculation, some of the treated animals (four out of ten) were even more susceptible to infection than the normal controls. Disregarding for the moment the therapeutic result in as far as the original infection is concerned, it is certain that this difference in the reaction of the treated and untreated animals can be attributed to no other cause than the treatment employed.

In the second place, it is practically certain that none of the animals was cured. In the majority of them, the original lesions were not completely resolved, but there still might be some doubt as to whether the relapses which occurred in the reinoculated animals were true relapses of the original infection or were lesions arising from the second inoculation. In several instances, the clinical history of the relapse appeared to be sufficient in itself to exclude the latter possibility, since within a time too short to permit of the development of metastatic lesions, reinduration and growth occurred in existing lesions. In order to remove any possible confusion which might arise from this source, however, fourteen therapeutic controls were set aside—infected and treated with the same material and in the same manner as the reinoculated animals—and none of these was cured.

The results of the experiments may, therefore, be reduced to a very simple statement; namely, that treatment of animals with marked primary lesions of the testicles altered their resistance to such an extent as to render them susceptible to a second cutaneous infection without having effected a cure of the original infection. It is clear, therefore, that under the circumstances existing in these experiments, not only is superinfection of rabbits possible but animals treated in the manner

described may be rendered even more subject to a new infection than a normal animal.

It should be emphasized that these findings do not conflict in any way with established facts of syphilitic immunity. The conflict, if there is one, is with the assumption that the same conditions obtain in treated as in untreated infections or that an immunity once established cannot be altered.

It has been clearly shown by the work of Finger and Landsteiner (2) and of others that immunity to a second infection is not always absolute—that by resorting to the use of large doses of virus, it is possible, even in advanced cases of syphilis, to produce superinfection, or lesions at the site of inoculation which tend to assume the form of those characteristic of the stage of infection during which reinoculalation is carried out. It is also well known that superinfection with the production of typical chancres is comparatively easy during the first incubation period, and multiple chancres and autoinoculation with chancre-like lesions are explainable upon this basis.

The facts enumerated have been found to apply to both human and animal infections. From the data available, the chief differences which appear to exist in untreated infections are that protection against reinfection develops much more quickly, is more marked, and more enduring in the rabbit than in man. These few facts furnish all the basis necessary for an understanding of the phenomena of a second infection following treatment which is not curative. It is only necessary to consider that treatment with such substances as arsphenamine and neoarsphenamine may cause an infection to revert to the condition which existed during the first incubation period when spirochetes were present but no immunity had developed; further, that the spirochetes which survive the action of the drug employed may be so attenuated or enfeebled for the time being as to be incapable of arousing an antagonistic reaction on the part of the host, thus favoring the propagation of more vigorous organisms introduced from without and the production of characteristic primary lesions at the site of the new infection.

These were the postulates which formed the basis for the experiments reported, and the results would appear to justify the conclusion that, in as far as early infections of the rabbit are concerned,

treatment with arsphenamine or neoarsphenamine may alter the immunological status of the animal to such an extent as to favor the propagation of a second infection without having accomplished a cure of the first. In advanced infections, conditions are more complicated, and it is more difficult to obtain chancres from second inoculations, but experiments now in progress indicate that even here superinfection is still possible of attainment.

Apart from any bearing which these experiments may have upon problems of syphilitic immunity, they serve to emphasize the necessity for a careful consideration of the influence which any therapeutic agent or any system of therapy may exert upon the mechanism of animal resistance as well as the spirocheticidal action of the agents employed.

CONCLUSIONS.

From the facts presented, it may be concluded that the existence of an infection with *Spirochæta pallida* does not constitute a bar in itself to the introduction and propagation of a second infection in the same animal; that just as there is a period following a first inoculation during which a second infection may be implanted with the production of characteristic primary lesions, conditions may again arise in animals which have once become refractory to a second inoculation, that will favor the introduction of a new infection with the formation of lesions presenting the characteristics of an original or first infection.

Experimentally, such a state may be induced in rabbits with early but well marked primary lesions of the testicles by treatment with either arsphenamine or neoarsphenamine, hence, treated but uncured animals may be rendered as susceptible to a second cutaneous inoculation as a normal animal, and the manifestations of disease resulting from the second infection may be indistinguishable from those of a first infection.

SUMMARY.

Experiments were carried out on rabbits for the purpose of determining the effects of subcurative doses of arsphenamine and of neoarsphenamine upon the resistance of infected animals to reinoculation with *Treponema pallidum* and hence the possibilities of the occurrence of a second infection in treated but uncured cases of infection.

All the animals used were inoculated with the same virus, and the experimental tests were carried out when the first cycle of testicular reaction was nearing its height. The animals with the most marked testicular lesions were used for the basic experiment of treatment and reinoculation. The results of this experiment were controlled from four different standpoints: (1) the effect of the treatment employed upon the existing infection; (2) the immunity present at the time of treatment; (3) the virulence of the organisms used for reinoculation as compared with those causing the existing infection; (4) the comparative susceptibility of normal animals to the virus used for reinoculation.

The results obtained showed (1) that the treatment employed was insufficient to cure any of the therapeutic controls; (2) that the infected controls were highly refractory to a second inoculation; (3) that the treated animals were highly susceptible to a second inoculation and although not cured of their original infection, reacted to the second inoculation with the formation of lesions indistinguishable from those of a first infection; (4) that in certain instances the treatment given had rendered infected animals more susceptible to infection than the normal controls.

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EXPLANATION OF PLATES.

The illustrations are reproductions of photographs which represent the objects at their natural size.

PLATE 73.

FIGS. 1 and 2. Reinoculation lesions of control animals.

FIG. 1. A lenticular papule at the base of the ear 15 days after reinoculation. The lesion did not progress beyond the point shown.

FIG. 2. 30 days after reinoculation. There is an indurated papule in the same location. These were the most marked lesions produced by reinoculation of the infected controls.

FIGS. 3 to 7. Reinoculation lesions following treatment with arsphenamine.

FIGS. 3 and 4. Characteristic chancres on the ear and sheath of a treated animal as they appeared 42 and 37 days after reinoculation.

FIGS. 5 and 6. Ear and sheath lesions of an animal with clinical relapse of the original lesions—37 days after reinoculation. The right testicle (Fig. 6) is diffusely enlarged and indurated and contains two well defined nodules; the left testicle is still atrophic but there is a small area of infiltration in the scrotum.

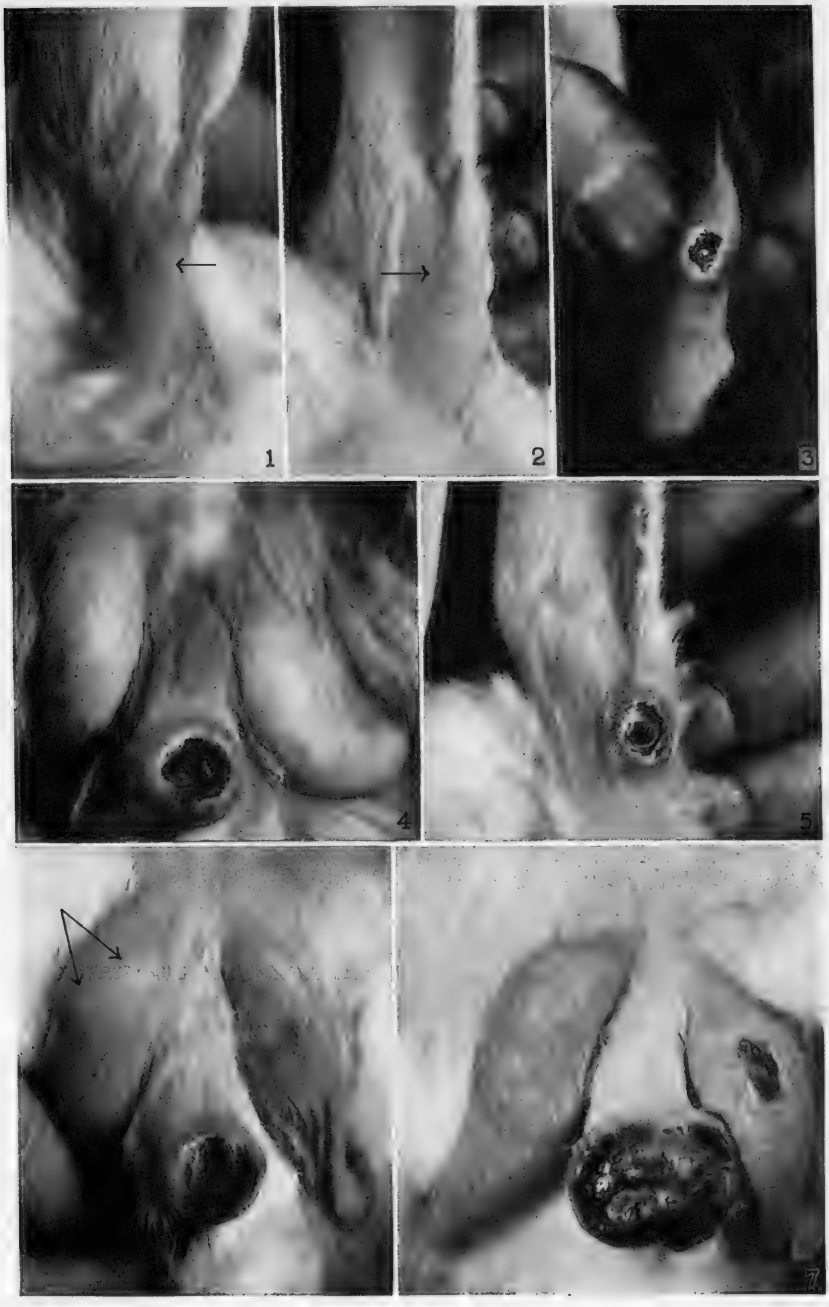
FIG. 7. The genital lesions of the same animal 12 days later. Note the marked increase of all lesions and the extensive necrosis of the lesion on the sheath.

PLATE 74.

FIGS. 8 to 12. Reinoculation lesions following treatment with neoarsphenamine.

FIGS. 8 and 9. These figures are from the same animal and represent conditions as they existed 42 days after reinoculation. The two lesions are about equally developed and show marked necrosis.

FIGS. 10 to 12. From a second animal. Figs. 10 and 11 are 42 days, and Fig. 12, 49 days after reinoculation.



(Brown and Pearce: Superinfection in experimental syphilis.)

(Brown and Pearce: Superinfection in experimental syphilis.)

FURTHER STUDIES ON THE BEHAVIOR OF BACTERIA TOWARD GENTIAN VIOLET.*

By JOHN W. CHURCHMAN, M.D.

(*From the Department of Bacteriology of Cornell University Medical College, New York.*)

PLATES 75 TO 77.

(Received for publication, November 3, 1920.)

Isolation of a Gentian-Positive Strain from a Culture of a Gentian-Negative Organism (a "Strain-within-a-Strain" Variant).

When divided gentian violet plates are stroked with broth cultures or with heavy suspensions of *Bacillus coli*, growth is equally good on both sides of the plate (Fig. 1). So, too, if divided plates are poured so that each half receives a heavy inoculation of *Bacillus coli*, numerous colonies appear in both halves, though a similar experiment with *Bacillus subtilis* shows complete sterility of the gentian violet side. If, however, the inoculations are made with increasingly weak dilutions of the suspension, the colonies gradually become fewer on the gentian violet side and finally disappear altogether (Fig. 2). This phenomenon is exhibited whether a 2 or a 24 hour culture is studied. It may be demonstrated as well by a slightly different type of experiment. If a plain agar plate is stroked with broth cultures of *Bacillus coli* which have been exposed to the dye, the stained organisms appear to grow as well as the controls, though strokes of stained Gram-positive organisms, for example, *Bacillus subtilis* or *Oidium albicans*, will not grow at all (Fig. 3). If, however, the experiment is repeated with increasingly weak suspensions of *Bacillus coli* it will be seen that by no means all the organisms have actually survived exposure to the stain (Fig. 4).

* A preliminary report of this work was published in the *Proceedings of the Society of Experimental Biology and Medicine* (Churchman, J. W., *Proc. Soc. Exp. Biol. and Med.*, 1920, xviii, 17, 19, 20, 21).

It is therefore possible, by stroking divided plates with weak dilutions of *Bacillus coli* to obtain plates, with a few colonies on the plain agar side and none on the gentian violet agar. Some of these colonies may be presumed to have sprung from individuals which would have grown on the gentian violet agar if they had happened to be implanted there, others may be presumed to be made up of organisms incapable of growing in the presence of the dye. By again stroking divided plates with suspensions of these separate colonies an opportunity was afforded of isolating a strain of *Bacillus coli* incapable of growing in the presence of the dye, unmixed with individuals capable of such growth. This has, indeed, been done.

The result of this experiment was to provide two strains—labelled for convenience Strains X and Y—isolated from a single "pure" culture of *Bacillus coli*. These two strains, both definitely Gram-negative, are identical by all the usual tinctorial, morphological, and cultural tests. Yet Strain X grows well, Strain Y not at all, or practically not at all in the presence of gentian violet (Fig. 5); and strokes of Strain X which have been exposed to the dye and then planted on plain agar grow equally well with the controls, while strokes of Strain Y made with organisms which have been exposed to the dye do not grow at all (Fig. 6).

That this specific characteristic is not transient is shown by the experiment represented in Figs. 5 and 6. This experiment was done after the two strains had been allowed to grow for 6 weeks through a number of transplants, and it will be seen that the original features of the two strains have been retained.

The experiments outlined above demonstrate that within a single bacterial strain two strains may exist which, though identical in morphology and in tinctorial and cultural reactions, are dissimilar in their reactions to gentian violet, one growing vigorously and the other not at all on media containing the dye. These two types retained their differential characteristics after many transplantations. This isolation, from a pure culture, of a "strain-within-a-strain" variant parallels the observation reported some years ago of a "strain-within-a-species" variant occurring in the *Bacillus enteritidis* group.[1]

[1] Churchman, J. W., and Michael, W. H., *J. Exp. Med.*, 1912, xvi, 822.

The distinction between the two types of variant strain is, of course, not fundamental; one type was encountered in nature, the other was isolated in the laboratory.

Explanation of the Parallelism between the Gram Reaction and the Gentian Violet Reaction.

While certain facts in the observations hitherto reported on the parallelism between the Gram reaction and the gentian violet reaction were difficult to explain on the theory that the two reactions depended on a common factor and that the parallelism was therefore a fundamental one, the preponderance of evidence pointed in this direction. About 90 per cent of the Gram-positive organisms are killed by exposure to the dye and will not grow on media which contain it; about 90 per cent of the Gram-negative organisms grow well after exposure to the dye, and their growth seems to be unimpeded by the presence of the dye in media.

That this difference between the effect of gentian violet on the Gram-positive and the Gram-negative organisms might be applied to the isolation of Gram-negative bacteria from mixtures with Gram-positive bacteria, and particularly to the elimination of contaminations with *Bacillus subtilis*, which so frequently prove troublesome, was pointed out in a previous publication.[2] The lead thus given has since received the following practical applications: (1) isolation of *Bacillus tuberculosis* from sputum and feces (Petroff[3]); (2) elimination of false presumptive tests in the examination of milk and water for *Bacillus coli*, by the use of gentian violet (Hall and Ellefson[4]); (3) selective elimination of hay bacillus from cultures of obligative anaerobes by the use of gentian violet (Hall[5]); (4) isolation of *Bacillus influenzæ* by the use of gentian violet (Bernstein and Loewe[6]); and (5) isolation of pathogenic moulds (*Epidermophyton inguinale*) by the use of gentian violet (Farley[7]).

[2] Churchman, J. W., *J. Exp. Med.*, 1912, xvi, 221.

[3] Petroff, S. A., *J. Exp. Med.*, 1915, xxi, 38.

[4] Hall, I. C., and Ellefson, L. J., *J. Bact.*, 1918, iii, 329, 355; *J. Am. Water Works Assn.*, 1919, vi, 67.

[5] Hall, I. C., *J. Am. Med. Assn.*, 1919, lxxii, 247.

[6] Bernstein, E. P., and Loewe, L., *J. Infect. Dis.*, 1919, xxiv, 78.

[7] Farley, D. L., *Arch. Dermatol. and Syphilol.*, 1920, ii, 466.

A parallelism so clear-cut and so amply confirmed could hardly be accidental, particularly as a similar parallelism was found to exist if the experiments were conducted with other dyes belonging to the triphenylmethane series. Moreover, it is just the constantly Gram-positive organisms like *Bacillus subtilis* which always fail to grow in the presence of even minute quantities of the dye, and just the constantly Gram-negative ones which grow readily in fairly strong dilutions of it (Fig. 1).[8]

The difference in avidity of the living organisms for the stain is also striking. *Bacillus subtilis* rapidly becomes deep violet and loses its motility; *Bacillus coli* or *Bacillus typhosus* stains slowly, only a small proportion of the individuals stain at all deeply, and all retain their motility for some time. If the Gram reaction is carried out with living organisms, centrifuged in a test-tube, the behavior of the negative and positive groups is the same as it is when they are killed and stained on a glass slide.

Facts of this kind led to the assumption that the parallelism between Gram reaction and gentian violet reaction depended on the presence in the Gram-positive organisms, and the absence from the Gram-negative ones, of unsaturated bodies avid for some portion of the triphenylmethane molecule. The saturation of these bodies would be thought of as accounting for the retention of the dye by the Gram-positive bacteria and their death in its presence; the absence of these bodies would explain why Gram-negative organisms fail to retain the dye and grow in media containing it.

There were, indeed, a few facts which would not conform to this hypothesis. There were in the first place the exceptional organisms; about 10 per cent of the Gram-positive strains studied were either unaffected by the dye or variable in their reactions to it, and an equal percentage of the Gram-negative cultures were somewhat susceptible to its bacteriostatic effects. Not only was this found to be the case in a study of all the common bacterial species, but an instance

[8] The chemical problems involved in the gentian violet reaction are now the subject of investigations in collaboration with Professor Bogert of Columbia University. It may be stated, however, that other members of the triphenylmethane series, which are themselves not dyes and possess relatively simple structural formulas, behave, as regards selective bacteriostasis, like gentian violet.

was encountered in which one strain of a species (*Bacillus enteritidis*) —and only one out of five studied—was quite sensitive to the bacteriostatic action of the dye, though the other four behaved quite according to rule, and all five were identical in cultural reactions and were definitely Gram-negative.[9]

An effort was made to test the fundamental nature of the parallelism between Gram and gentian reactions by training a Gram-positive and gentian-positive organism (*Bacillus subtilis*) to grow in the presence of the dye. If such an organism could be made in this way to lose its gentian positiveness without changing its Gram positiveness it would be clear that the two reactions did not depend on a common factor. If, on the other hand, both reactions changed, the opposite would be true.

The efforts to train *Bacillus subtilis* to grow in gentian violet were wholly unsuccessful. This organism will not grow on divided plates containing the dye in strengths of 1:1,000,000. It is possible, it is true, to induce it to grow in slightly stronger dilutions. But not much advance can be made, and the advance made is not retained; for the few organisms which have appeared in the stronger dilutions, if transplanted, fail to grow except in the weaker dilutions, around 1:1,000,000.

The problem was, however, solved in an entirely different way. If thick suspensions of the Gram-negative and gentian-negative *Bacillus coli* are stroked across a divided gentian violet plate, growth is equally vigorous on the two sides (Fig. 1). If, however, increasingly weak dilutions of the suspension are used for the stroking, the colonies on the gentian violet side of the plate become rapidly fewer (in proportion to those on the plain agar) as the dilution increases, and finally disappear altogether (Fig. 2). This is due, in part at least, to the fact, entirely obscured when thick suspensions are used for the experiment, that only a relatively small proportion of the

[9] It is interesting to note that Bronfenbrenner and his coworkers (Bronfenbrenner, J., Schlesinger, M. J., and Soletsky, D., *J. Bact.*, 1920, v, 24) in a study of the bacterial action of the CR indicator (China blue-rosolic acid) tested out this strain of *B. enteritidis* and found it to be exceptional also in its behavior toward CR. I have found that rosolic acid possesses selective properties similar to those of gentian violet.

individuals in a suspension of a Gram-negative organism are really gentian-negative; the majority will not grow in the presence of the dye.[10] If the suspension used for stroking the plate is thick, this small proportion of gentian-negative individuals is (absolutely) sufficiently large in quantity to produce good growth in the presence of the dye. If, on the other hand, the suspension is weak the gentian-negative individuals are, not only relatively, but absolutely few in number; few colonies therefore appear on the violet agar; if the dilution is very weak, none appears.

It is not difficult to isolate in pure culture the individuals which will and those which will not grow in the presence of the dye (Figs. 5 and 6). When so isolated the gentian-positive strain, that is the strain which will not grow in the presence of the dye, is found to be as definitely Gram-negative as the gentian-negative strain. The factor which determines the reaction of an organism to the Gram process of staining is not therefore the same as the factor which determines its growth reaction in the presence of gentian violet.

Effect of Repeated Reinoculations of Gentian Violet Agar with a Gentian-Positive Organism.

If a divided gentian violet plate containing the dye in a dilution of 1:100,000 is stroked with a thick suspension of the Gram-positive and gentian-positive *Bacillus subtilis*, no growth will ever occur on the gentian violet side of the plate; the organism will, indeed, fail to grow up to the dividing line between plain and gentian violet agar, ceasing sharply at a point 0.5 to 1 cm. from this line (Fig. 1). This inhibition takes place even when the dye is present in extremely weak dilutions. Growth is prevented by dilutions of 1:1,000,000 (Fig. 7), inhibited by 1:2,000,000, and only becomes vigorous at a strength of 1:3,000,000.

If, however, the gentian violet half of the plate is repeatedly and heavily reinoculated on successive days in the same place, a fair growth,—in some instances a rather vigorous growth—may finally

[10] It cannot be said, since the whole bacterial field has not been studied with this point in view, that all Gram-negative species contain these two strains. The specimen of *B. coli* with which the experiments were done and the specimen of *B. typhosus* and *B. prodigiosus* examined contained them.

be obtained. In Fig. 8 such an experiment is shown. Two strokes of a thick suspension of *Bacillus subtilis* were made on this plate. Nothing further was done to the right-hand stroke and the usual effect of the dye is clearly shown. In the left-hand stroke from the edge where the original growth on the plain agar ceased (this edge will be readily recognized), organisms were repeatedly resmeared over the gentian violet agar; a fair growth resulted.

That this growth which results from restroking is not due to acclimatization of organisms to the dye seems likely from the fact that prolonged attempts at experimental acclimatization of *Bacillus subtilis* to gentian violet have been unsuccessful. Furthermore, positive proof that acclimatization is not the explanation is given by the fact that if organisms which have, by reinoculation, been thus induced to grow on gentian violet agar, are freshly smeared on another divided plate they will not grow on the side of the plate which contains the dye. This phenomenon can hardly be due to insufficiency of dye in the presence of increase of inoculated organisms, for in the plates used in the experiments dye was present in strengths (1: 100,000) greatly in excess of the strength necessary to prevent the growth of *Bacillus subtilis* (1:1,000,000); and owing to the great diffusibility of the dye through agar much of that present in the plates is available for the organisms.

Whether the phenomenon is due to a change in the dye caused by organisms which, though not surviving, live sufficiently long to prepare the soil for subsequent implants; whether it is due to some protective barrier or nutritional or growth-accelerating substance provided by the dead bodies of bacteria to the living bacteria which rest upon them in subsequent seedings; or whether it is due to a communal activity of organisms—to which further reference is made in the following paper—are questions at present under experimental study. But the fact itself is sufficiently significant for certain problems in the chemotherapy of infections.

In searching for a possible explanation for these facts it is important that attention should be called to experiments which demonstrate the effect of the presence of dead bacterial bodies in media on which transplants are made. An experiment of this sort is illustrated in Figs. 9 and 10. The tubes contained gentian violet agar.

The surface of the control tube (Fig. 9) was inoculated with *Bacillus subtilis* and, as this organism never grows in the presence of the dye, no growth occurred. The surface of the other tube (Fig. 10) was covered with a thin layer of killed, washed organisms[11] (*Bacillus subtilis*). After this layer dried an inoculation of living *Bacillus subtilis* was made on top of it. Growth occurred, owing apparently to a complete protection of some sort interposed by the dead bodies between the living bacteria and the dye, or to some effect of the dead bacteria on bacterial growth. A similar result is obtained if dead bodies of *Micrococcus aureus* or of *Bacillus coli* are smeared on the surface of the gentian violet agar instead of dead bodies of *Bacillus subtilis*. Such an experiment, besides bringing out a fact to which attention has not hitherto been directed, shows clearly that dead bacterial bodies lying in a wound may entirely alter the reaction between a bacteriostatic agent and the living organisms which one is attempting to reach.

Selective Activity of Gentian Violet in Relation to Chemotherapy.

That the selective bacteriostatic action of gentian violet, demonstrable *in vitro*, is capable of clinical application in the treatment of infections has been amply demonstrated by experiments conducted in the hospital wards during the last 6 years. It has been shown that in certain types of acute infections of joints, staining of the synovial membrane (after proper mechanical cleansing by special methods) leads to prompt sterilization and clinical cure.[12] It has also been shown that with this dye it is possible to rid granulating wounds of

[11] The organisms were grown on medium containing only distilled water and agar, in order that their bodies might contain the minimum amount of nutritive substance. These bacterial bodies were washed six times with distilled water and were killed. Suitable controls were made to insure sterility. In order to prove that no available food material was still present, a control experiment was done in which these dead bacterial bodies were smeared on the bottom of a Petri dish, dried, and then inoculated with live *B. subtilis*, suitable moisture being provided. No growth occurred on incubation. The growth of *B. subtilis* in the experimental tubes illustrated in Fig. 10 was not, therefore, due to any nutritive material carried over in the dead bacterial bodies.

[12] Churchman, J. W., *J. Am. Med. Assn.*, 1918, lxx, 1047; 1919, lxxii, 1280; 1920, lxxv, 583.

certain organisms which have resisted other bacteriostatic agents; this was proved by the successful treatment of two patients with thigh amputations at the Walter Reed Hospital. The stumps of these individuals had become diphtheria carriers, but by using the dye they were rid of organisms which had persisted for weeks in spite of all treatment.[13] That the selective power of the dye, so clearly shown in divided plates, would also be demonstrated by a study of its effects in wounds was well shown during these efforts to sterilize infected amputation wounds. *Bacillus diphtheriæ* was caused to disappear without much difficulty, but in a stump infected with *Bacillus coli* it was impossible to rid the wound entirely of its organisms.

In spite of this considerable amount of clinical success in the treatment of a certain type of infection with gentian violet, the observations reported in the previous communications indicate that selective bacteriostasis is a very complex process and point out the difficulties attending the direct transfer of laboratory findings into therapeutics. The method of divided plates presents an excellent means of studying the interaction between dye and organism; the isolation of a pure strain of gentian-negative *Bacillus coli* makes it possible to investigate the question by single cell experiments of a peculiarly reliable kind; and the selective property of gentian violet may be taken advantage of for the study of the various elements of selective bacteriostasis on a single plate. Such a plate is represented in Fig. 11. This experiment was done to demonstrate the futility of generalizations about the effect of a chemotherapeutic agent on bacteria, even when the generalization is confined to its effect *in vitro*, and to indicate the necessity of bearing in mind, when drawing conclusions from experiments in this field, the conditions under which the experiment was done. On this plate, as will be seen (Fig. 11) seven different effects of gentian-violet on bacteria are shown, the result obtained depending on the organism chosen for the experiment and the conditions under which the inoculation was made. These different results are as follows: (1) Thick suspensions of Gram-positive organisms (*Bacillus subtilis*) will not grow in the presence of the dye (Fig. 11, *A*). (2) By repeated reinoculations of these organisms a moderate

[13] Churchman, J. W., *J. Am. Med. Assn.*, 1920, lxxiv, 145.

growth can be procured on the gentian violet agar (Fig. 11, *B*). (3) Thick suspensions of the Gram-negative *Bacillus coli* grow equally well on the two halves of the plate (Fig. 11, *C*). (4) If weak dilutions of a suspension of *Bacillus coli* are stroked across the plate almost no growth occurs on the gentian violet side (Fig. 11, *D*). (5) A thick suspension of the gentian-negative strain of *Bacillus coli* grows equally well on the two halves of the plate (Fig. 11, *E*). (6) If a weak dilution of the gentian-negative strain of *Bacillus coli* is stroked across the plate no growth may occur on the gentian violet side (Fig. 11, *F*). (7) If a thick suspension of the gentian-positive strain of *Bacillus coli* is stroked no growth occurs on the gentian violet agar (Fig. 11, *G*).

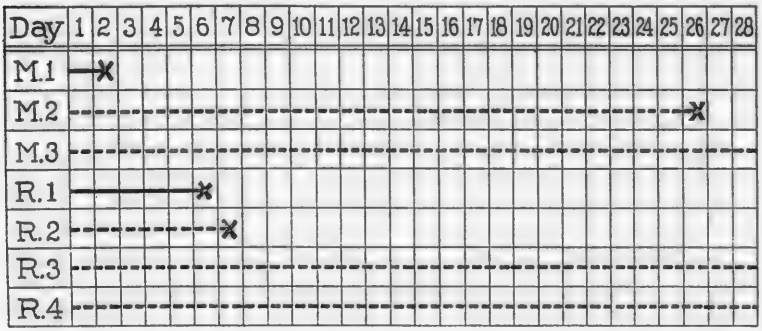
Unless considerations of this kind are borne in mind one may easily fall into gross error. For example, if the original experiments had been conducted with a thin suspension of *Bacillus coli* and the result seen at *D*, Fig. 11, had been obtained the conclusion would have been drawn that gentian violet has a strong bacteriostatic effect on *Bacillus coli;* whereas if thick suspensions are used, the opposite proves to be the case (Fig. 11, C). This error has, as a matter of fact, been committed by certain observers working with other dyes.

Since the single cell method of Barber[14] offers an important method for the study of reactions between chemical substances and single bacteria, it cannot be too strongly emphasized that the effect of a bacteriostatic agent on a group of bacteria may be quite different from its effect on individual organisms.

That the effect of a chemotherapeutic agent should be conceived in terms of stasis rather than death has been proved by the whole trend of the work with gentian violet. The difficulty of proving that organisms which have apparently been killed by chemical agents are really dead may be greater than might at first be supposed; and in any experiments in this field it is well to remember that organisms apparently killed may survive and give signs of life after a long period of lag. A striking instance of this phenomenon is shown in Text-fig. 1. This experiment was one of a series done to determine by animal inoculations whether stained gentian-positive organisms, which do

[14] Barber, M. A., *Philippine J. Sc.*, 1914, ix, 307.

not grow in culture media, are actually killed by exposure to the dye. Two series of animal inoculations—one with *Staphylococcus aureus* and the other with a strain of *Blastomyces* pathogenic for guinea pigs —seemed to warrant the conclusion that the stained organisms were killed, for all the animals that received unstained organisms died of the disease, and all those that received stained organisms survived. With stained *Bacillus anthracis*, however, different and striking results were obtained. This organism will not grow when it is planted on agar after having been stained. When injected in large amounts,



TEXT-FIG. 1. Results of animal inoculation with stained *B. anthracis*. *M.*, mouse; *R.*, rabbit. The course of control experiments is represented by a solid line, the course of experimental animals by a broken line. *X* indicates that cultures made at death from the heart's blood and spleen were positive for *B. anthracis*. Mouse 3 and Rabbits 3 and 4 survived without symptoms. Mouse 2 died of anthrax on the 26th day; the blood and spleen swarmed with the organisms.

after staining, into the susceptible mouse, it often causes death, though not infrequently the inoculated animal lives. But the point worthy of note is that when the animal dies it is sometimes after a period of many days of perfect health, though the control mice die in 36 hours. Mouse 2, for example (Text-fig. 1) died of anthrax on the 26th day, having been in perfect health in the interval; that is, it behaved as if it had received the injection more than 3 weeks after the inoculation had actually been made. The stained organisms, nearly but not quite killed by their exposure to the dye, had suffi-

ciently recuperated at the end of 26 days to bring about the death of the animal from anthrax. In the animals which survived the inoculation entirely it is not unlikely that the defensive mechanism of the body was successful in overcoming the bacteria, which were injured but not killed. The experiments prove that the survival of injected animals does not necessarily prove that the organisms injected were dead.

SUMMARY.

1. A gentian-positive strain (a "strain-within-a-strain" variant) has been isolated from a pure culture of a gentian-negative organism. This observation corresponds to that of a "strain-within-a-species" variant, occurring in the *enteritidis* group, reported some years ago.

2. The Gram reaction and the gentian reaction do not depend, as has been assumed in previous publications, on the specific affinity of the gentian-positive organisms for a portion of the gentian violet molecule, since certain Gram-negative strains are shown to be gentian-positive.

3. Dead bacterial bodies interposed between living bacteria and gentian violet media partially negative the effect of the dye on Gram-positive organisms and allow them to grow. This seems to be either a phenomenon of filtration or of stimulation of growth.

4. The application of these facts by the method of divided plates shows a number of difficulties in the application of laboratory studies to chemotherapeutics, which would escape observation by ordinary methods.

EXPLANATION OF PLATES.

PLATE 75.

FIG. 1. Divided gentian violet plate (gentian violet 1:100,000). *T*, *B. typhosus; S*, *Micrococcus aureus; OA*, *Oidium albicans; Su*, *B. subtilis; C*, *B. coli.* It is the strongly Gram-positive organisms that fail to grow and the strongly Gram-negative ones that grow.

FIG. 2. 24 hour cultures of *B. coli.* Disappearance of all colonies from the gentian violet agar side of the plate as the dilution of suspension used for stroking becomes weaker. *C*, *B. coli*; *Broth*, broth culture (undiluted).

PLATE 76.

FIG. 3. Effect of staining on Gram-negative *B. coli* and Gram-positive *Oidium albicans* (*cf.* Fig. 4). *C*, unstained *B. coli* (control); *C'*, stained *B. coli; O*, unstained *Oidium albicans* (control); *O'*, stained *Oidium albicans.*

FIG. 4. Effect of staining increasingly weak dilutions of a suspension of *B. coli* with gentian violet. *Con*, unstained organisms; *St*, stained organisms.

FIG. 5. Behavior of Strains X and Y on a divided plate 6 weeks after first isolation.

FIG. 6. Behavior of Strains X and Y on exposure to gentian violet 6 weeks after first isolation. Plain agar plate. *X*, unstained organisms (control); *X'*, stained organisms. *Y*, unstained organisms (control); *Y'*, stained organisms.

FIG. 7. *B. subtilis* on a divided plate. The upper half contains gentian violet in 1:1,000,000 dilution. (*A* refers to the series of experiments.)

FIG. 8. Two strokes of a thick suspension of *B. subtilis* were made. On the right nothing further was done. On the left reinoculations were made on the gentian violet agar.

PLATE 77.

FIG. 9. Implantation of *B. subtilis* on the surface of gentian violet agar; no growth resulted. *a*, site of inoculation.

FIG. 10. Implantation of *B. subtilis* on gentian violet agar the surface of which had been covered with a thin layer of killed, washed organisms. *a*, colony of *B. subtilis; b*, layer of killed washed organisms (*B. subtilis*); *c*, photograph of the surface of the tube, showing the colony of *B. subtilis.*

FIG. 11. The figure shows how the result of an experiment in glass on the effect of a bacteriostatic agent may vary with slightly changing conditions and illustrates some of the difficulties encountered. *A*, stroke of a suspension of *B. subtilis; B*, stroke of a suspension of *B. subtilis* with reinoculation of gentian violet agar; *C*, stroke of a thick suspension of *B. coli; D*, stroke of a thin suspension of *B. coli; E*, stroke of a thick suspension of Strain X; *F*, stroke of a thin suspension of Strain X; *G*, stroke of a thick suspension of Strain Y.

FIG. 1.

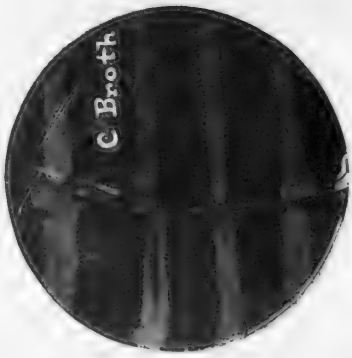


FIG. 2, *a*.

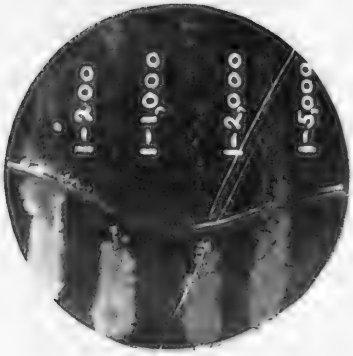


FIG. 2, *b*.

FIG. 2, *c*.

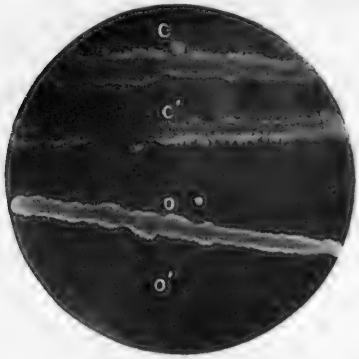


FIG. 3.

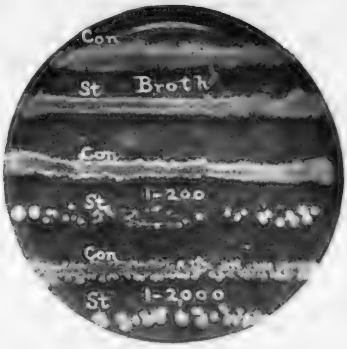


FIG. 4.

FIG. 5.

FIG. 6.

FIG. 7.

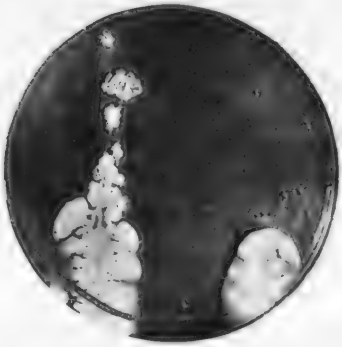
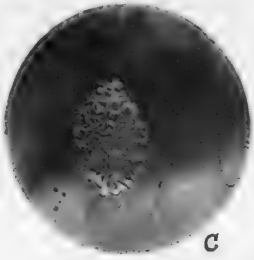


FIG. 8.

(Churchman: Behavior of bacteria toward gentian violet.)



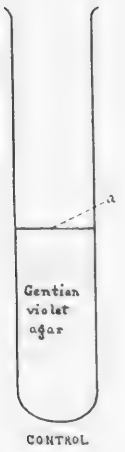


FIG. 9.

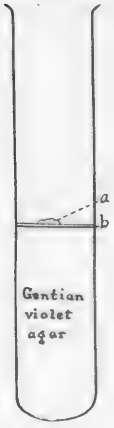


FIG. 10.

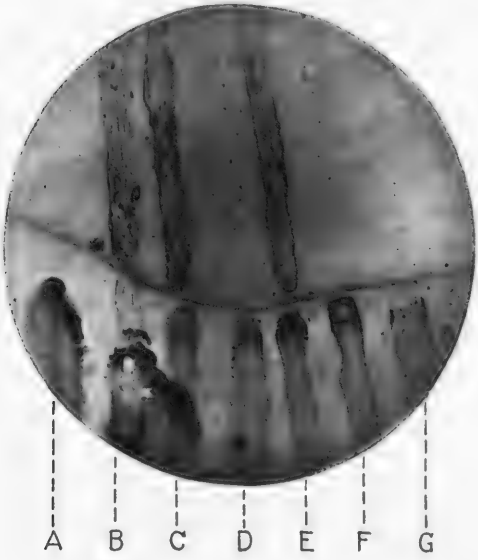


FIG. 11.

(Churchman: Behavior of bacteria toward gentian violet.)

COMMUNAL ACTIVITY OF BACTERIA.*

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Plates 78 to 80.

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The isolation of a strain of *Bacillus coli* fast to gentian violet, that is one containing no individuals susceptible to the bacteriostatic properties of the dye,[1] has made it possible to study quantitatively the reaction between this bacteriostatic agent and bacteria, without encountering the disturbing factor usually met in such studies which results from the variability in susceptibility of individual organisms to the chemical substance under examination. This strain —Strain X—had been isolated from a single colony growing on gentian violet agar, and had been kept growing, by frequent transplants, on media containing the dye, over a period of several weeks.[2] The ability of every individual to grow in the presence of the dye had therefore been proved.

With this strain a large number of single cell and small group transplants have been made in order to see whether any difference could be observed between the behavior of isolated individual organisms and that of very small aggregations of the same organisms. The experiments showed that a marked difference exists.

The technique used was that of Barber.[3] Only motile organisms were transplanted. The transplants were always made with young broth cultures 2 to 4 hours old, but it was found equally important to use for the stock cultures, from which the broth cultures for study were made, fresh agar transplants not more than 18 hours old; when

* A preliminary report of these observations was published in the *Proceedings of the Society of Experimental Biology and Medicine* (Churchman, J. W., *Proc. Soc. Exp. Biol. and Med.*, 1920, xviii, 22).

[1] Churchman, J. W., *J. Exp. Med.*, 1921, xxxiii, 569.

[2] See Figs. 5 and 6, of the preceding paper.[1]

[3] Barber, M. A., *Philippine J. Sc.*, 1914, ix, 307.

older agar transplants were used there were delay and inconstant results in the controls. We observed this type of lag, due to the age of the culture from which the subculture for the transplantation was made, independently, before knowing of Chesney's work.[4] To bear it in mind in single cell work is essential.

To determine the behavior of single cells in the presence of gentian violet, transplantations were made onto gentian violet agar and into gentian violet broth, and by way of comparison, similar transplants of small groups of cells were made into the same media.

Transplantation onto Gentian Violet Agar.

Although, as has been said, the organism with which the experiments were done was definitely gentian-negative and grew with apparently no inhibition when strokes of a heavy suspension were made onto agar containing the dye,[5] transplants of single cells almost never grew, although as high as 85 per cent positives were obtained in the controls. In the only two instances in the whole series of single cell transplants in which growth occurred, marked delay took place, a delay which was never observed in the controls. Moreover, transplants of small groups of organisms (five to fifteen) did not grow, though transplants of thirty individuals grew regularly (Figs. 1 to 5).

Transplantation into Gentian Violet Broth.

The experiments just cited made it seem likely that there was some fundamental difference between the behavior of a single cell and that of a small group of cells in the presence of gentian violet and this probability became a certainty as a result of the transplantations made into gentian violet broth in a dilution of 1: 100,000, pH 7.2. The gentian negativeness of the organism used for this series of experiments was even more authentic than that of the one used in the agar experiments. It came from the colony marked *B* in Fig. 5. This was one of the only two colonies which ever appeared in our experiments after transplantation of a single cell onto gentian violet agar. Colony A on this plate was just visible at the end of 18 hours, though

[4] Chesney, A. M., *J. Exp. Med.*, 1916, xxiv, 387.

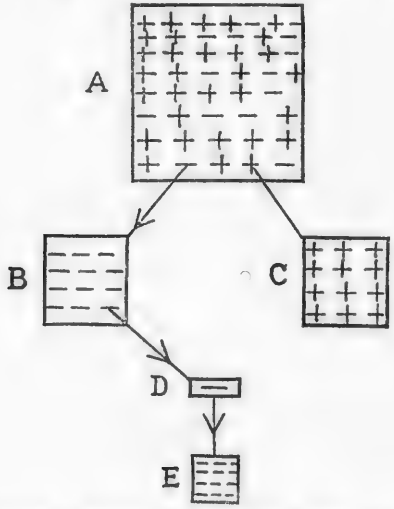
[5] See Fig. 5 of the preceding paper.[1]

the controls were well developed at this time (Fig. 3). At the site where Colony B subsequently developed nothing was at this time to be seen, even with a magnifying glass; Colony B appeared during the second 24 hours.

The organisms in Colony B provided material for a study of the effect of gentian violet on single cell transplants that was above criticism. Not only had it come from a pure gentian-negative strain and been kept growing for weeks on gentian violet media, but the organisms in Colony B were all the descendants of a single cell which had survived and reproduced in the presence of the dye. One could feel certain that every individual in this culture was gentian violet-negative; one did not have to consider the possibility that failure of growth in single cell transplants might be a matter of chance and due to the presence of individuals susceptible to the dye, which happened to be picked up. As a matter of fact, this possibility of a chance picking up of susceptible organisms could hardly be seriously considered as an explanation of the results, if the large number of transplants made is borne in mind.

The pedigree of Colony B is shown in Text-fig. 1. The results of the inoculation of broth with this strain which was called Strain Z showed beyond doubt that there is a fundamental difference between the behavior of one cell and that of a small group of cells toward gentian violet. 80 per cent positives were obtained in the controls in which single cells were inoculated into plain broth; and almost 100 per cent positives were obtained when thirty or more cells were planted in gentian violet broth. Yet when single cells were planted in gentian violet broth, or when very small groups (two to eight organisms) were seeded, growth did not occur (Figs. 6 and 7). On the basis of 140 successive single cell transplants into gentian violet broth without growth in any instance, the conclusion seemed justified that single cells would never grow under these conditions. In a final series, however, of eight inoculations delayed growth was obtained in one tube in the second 24 hours, an occurrence so rare that it seems justifiable to regard it as the occasional and unexplained exception met with in the study of almost every biological phenomenon. These negative results with single cells were striking when compared with the almost absolute constancy with which growth occurred when groups of thirty or more cells were inoculated.

The term communal activity of bacteria is used to express the facts just detailed. This term is open to the objection that bacterial interreactions are implied, for which there is at present no rigorous evidence. It might be that thirty cells succeed in growing, merely because thirty cells are able to produce some antidye substance in an amount sufficient to destroy the bacteriostatic effect of the dye, while one cell fails to grow because it is unable to do this. Without



TEXT-FIG. 1. Pedigree of Strain Z. *A*, original culture of *B. coli*, containing gentian-negative and gentian-positive individuals; *B*, pure culture of gentian-negative strain, isolated from *A; C*, pure culture of gentian-positive strain isolated from *A; D*, single cell from Strain B; *E*, colony resulting from planting Strain D on agar.

implying anything as to the underlying source of the phenomenon we use the term communal activity to describe it in order to indicate that in the contest between bacteria and bacteriostatic agents very small groups of organisms may be able to accomplish together what they could not accomplish if working singly. The significance of these facts for the study of chemotherapy and particularly for the study of the effect of bacteriostatic agents by the single cell method is clear. One cannot conclude from the behavior of a single cell in the

presence of such a substance anything as to the behavior of a group of cells, even a very small group, under the same conditions.

It may perhaps be again emphasized that the results obtained in these experiments were clearly not due to individual variants, susceptible to gentian violet, which happened to be picked up in making the single cell transplants. Not only was the strain used one which had proved its insusceptibility to gentian violet by growing in the presence of this dye, but the failure of growth in 140 consecutive single cell transplants into gentian violet broth eliminates, by the law of probabilities, such an explanation.

It should be clear that the fact here established is of a different order from the well known relation between growth and gross size of inoculum. The presence of large numbers of dead organisms in the ordinary culture and of organisms which, though living, are susceptible to the slightly unfavorable conditions of the new media into which they are transplanted makes it more probable that growth will follow the inoculation of media with 500,000,000 organisms than if only 500 are used. But in the experiments described here only living, motile organisms were used, and only individuals from a strain which had proved its ability to grow in the presence of the dye and did so constantly when inoculated in groups of thirty or more. It seems probable that some factor not hitherto recognized must be found to account for the difference in behavior between one cell and thirty cells and that it is a factor other than that which accounts for the difference in behavior between 500 cells and 500,000,000.

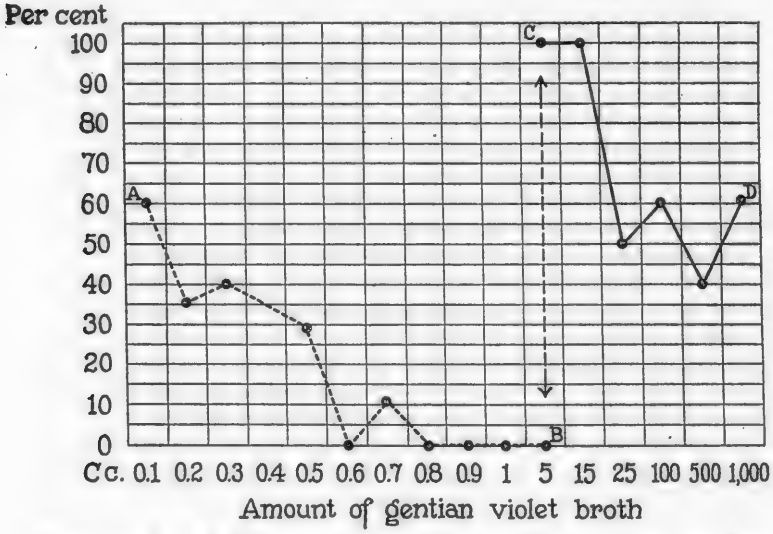
We have done a large number of experiments to determine whether the facts just detailed were to be explained simply by the relation of the number of transplanted organisms to the amount of available gentian violet; that is, whether the whole phenomenon is a purely quantitative one. Large inoculations of this gentian-negative strain grow in the presence of gentian violet without any apparent restraint; so, too, do inoculations of thirty cells; whereas single cells, under identical conditions, do not grow at all. This might be due to the fact that groups of cells, even small groups of thirty individuals, were able to make some change in the dye, gentian violet being assumed to offer a slightly unfavorable medium even for this gentian-negative strain, in spite of the absence of any apparent inhibition to the growth

of groups of cells. Single cells might be unable to effect this change in dye in an amount sufficient to allow growth to take place. If this explanation were the correct one, it should be possible to demonstrate an approximate relation between the number of organisms and the amount of gentian violet broth in which a given group could grow. In the experiments thus far described the transplants were made into 5 cc. of 1: 100,000 gentian violet broth. Under these conditions one cell would not grow but thirty cells would. If it is assumed that this was because thirty cells could produce thirty times the amount of antidye substance produced by one cell, then by merely multiplying by thirty the amount of gentian violet broth into which the inoculations were made, that is by using for example 150 cc., the growth of thirty organisms should be prevented. As a matter of fact thirty organisms will grow, with a fair degree of constancy, not only in this amount of gentian violet broth, but in very much larger amounts. When inoculations of thirty organisms were made, for example, into a liter of gentian violet broth, growth occurred in 60 per cent of the flasks, though if the explanation of the phenomena described were purely quantitative it should be necessary to seed 6,000 organisms into this amount of gentian violet broth in order to obtain growth; that is, thirty organisms accomplish not thirty times what one organism accomplishes, but very much more than this. This discrepancy between the work actually accomplished by thirty organisms, and the work which they might be expected to accomplish on a purely quantitative basis is represented by the line *A–B* in Text-fig. 2.

It might be objected that bacteria ought not to be thought of as units exactly equal to one another in efficiency. A given group of thirty might be able to do only fifteen times the work of one very vigorous organism, while another group of thirty might be able to do 60 times the work of a relatively weak organism. If, however, there was any very marked individual variation in the ability of individual organisms to cope with gentian violet, this would have shown itself in occasional growths of vigorous cells when single cell transplants were made. Furthermore, such a variation would hardly explain why thirty cells can grow in a liter of gentian violet broth, and thus do what not less than 6,000 might be expected to do.

TEXT-FIG. 2. The solid line at the bottom of the chart represents the curve of growth of thirty organisms. These grow even in a liter of gentian violet broth. The dotted line represents the curve that would be expected if the growth of groups of organisms depended entirely on the quantity of available gentian violet; in a liter of gentian violet broth 6,000 organisms would have to be seeded to produce growth. The line *A–B* represents the discrepancy between what actually occurs and what would be expected to occur from a quantitative theory of the difference between the behavior of one cell and that of a group of cells.

That the amount of available gentian violet does, within very narrow limits, play some part in the prevention of growth of single cells is shown in Text-fig. 3. The dotted line A–B represents the results of single cell inoculations into gentian violet broth. It will be seen that single cells never grow in more than 0.8 cc., but that in amounts smaller than this a fair number of positives occur. When 0.1 cc. was used 60 per cent positives were obtained. The corresponding amount of gentian violet broth for thirty organisms would



TEXT-FIG. 3. *A–B* is the curve of growth for single cells—0 per cent when 5 cc. of broth were used, 60 per cent when 0.1 cc. was used. *C–D* is the curve of growth for thirty cells, 100 per cent when 5 cc. of broth were used, 60 per cent when 1 liter was used.

be thirty times 0.1 cc., or 3 cc.; yet as a matter of fact 60 per cent positives were obtained with thirty organisms in over 300 times this amount of gentian violet broth; that is, in a liter (Text-fig. 3, line *C–D*). It is important to observe that the amount of broth used for the experiments, independent of the amount of gentian violet, did not play a part in the results, for in experiments made to determine this point, 75 per cent positives were obtained when single cells were inoculated into a liter of plain broth.

It seems clear, therefore, that thirty cells, instead of being able to accomplish thirty times what one cell can accomplish, are able to accomplish very much more than this. To this discrepancy between the work that thirty cells can do and the work they might be expected to do, on a purely quantitative basis, the term communal activity has been applied. For the present its nature cannot be more accurately defined.

SUMMARY.

1. The behavior of a single bacterial cell toward gentian violet differs fundamentally from that of a small group of cells (thirty).

2. The explanation of this phenomenon is not purely quantitative; thirty cells accomplish much more than thirty times what one cell can accomplish.

EXPLANATION OF PLATES.

PLATE 78.

Fig. 1. Single unstained cells planted on gentian violet agar; no growth.

Fig. 2. Groups of unstained cells of a gentian-negative strain of *B. coli* planted on gentian violet agar. The smaller groups did not grow. The number of cells in the group planted is indicated on each tube.

PLATE 79.

FIG. 3. Transplantation of single cells on plain agar for control. In Divisions 1 and 2 two separate plants (*a* and *b*, *c*, and *d*) were made, in the other divisions only one. Six growths were obtained out of seven plants.

FIG. 4. Transplantation of single cells of *B. coli* on gentian violet agar. One cell was planted in each of the ten divisions. No growth occurred.

FIG. 5. Transplantation of single cells of *B. coli* on gentian violet agar. A single cell was planted in each division. *A*, barely visible, *B*, invisible at the end of 24 hours. These two colonies are the only two growths which have ever occurred from single cells in gentian violet media.

PLATE 80.

FIG. 6. Single cell transplantations into broth. Strain Z, a gentian-negative strain from a single cell, was used for the seeding. Growth is represented by shading. 80 per cent positives were obtained in the control series (upper row), no positives in the gentian violet broth series (lower row).

FIG. 7. Group transplantations of a gentian-negative strain of *B. coli* (Strain Z) into gentian violet broth. The number of cells seeded is indicated on each tube. Groups below twenty-seven did not grow. Compare the growth of the larger groups with the failure of single cells to grow (see Fig. 6).

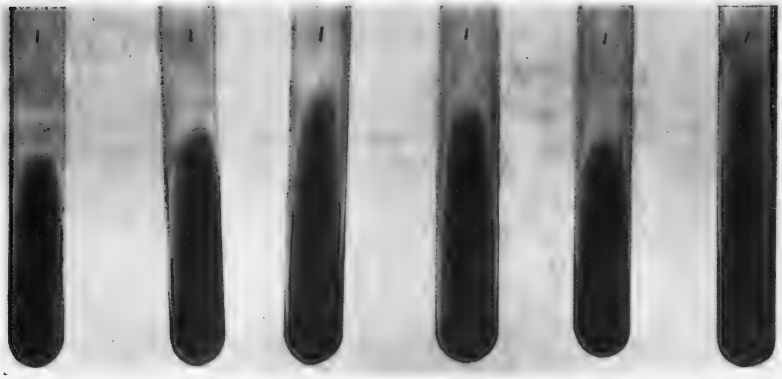


FIG. 1.

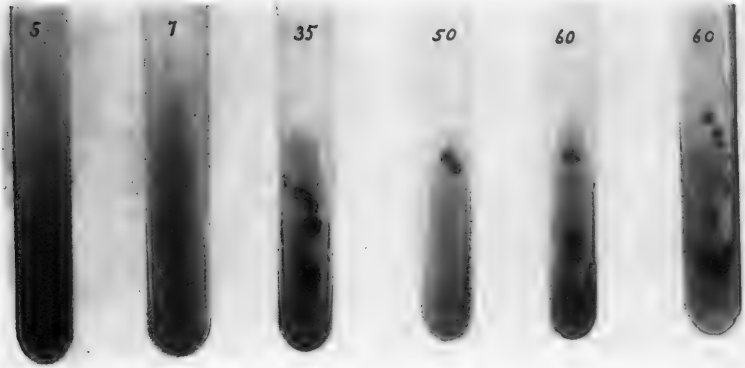


FIG. 2.

(Churchman and Kahn: Communal activity of bacteria.)

FIG. 3.

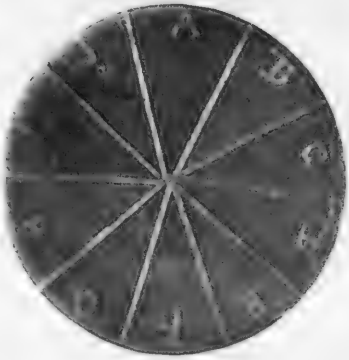


FIG. 4.

FIG. 5.

(Churchman and Kahn: Communal activity of bacteria.)

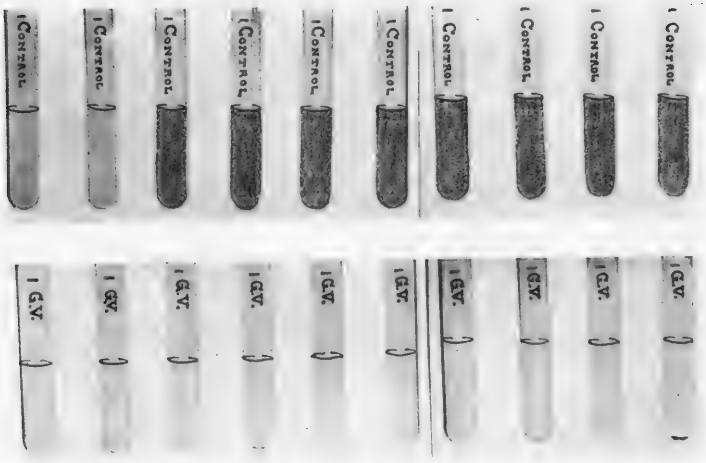

1 Control
1 G.V.

FIG. 6.

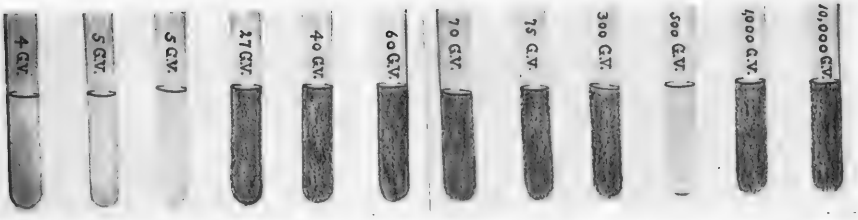

4 G.V.
5 G.V.
5 G.V.
27 G.V.
40 G.V.
60 G.V.
70 G.V.
75 G.V.
300 G.V.
500 G.V.
1000 G.V.
10,000 G.V.

FIG. 7.

FATE OF THE LYMPHOCYTE.

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The normal function of the lymphocyte, as a cell, and the fate of the lymphocytes that enter the blood circulation are questions which apparently have not been solved. It seems to be generally accepted that lymphocytes are produced in the lymphoid tissue of the body and only to a minor extent, if at all, in the bone marrow. From the lymphoid tissue they gain entrance to the efferent lymphatics, and are, in the words of Davis and Carlson,[1] "as much a part of the lymph as the erythrocytes and leucocytes are of the blood." Eventually the lymphocytes, which have entered the lymph stream, in part at least, reach the blood circulation through the major lymph trunks, and chiefly through the thoracic duct. The content of the thoracic duct lymph is high in lymphocytes. Thus, in eleven dogs studied by Rous[2] the leucocytes per c. mm. varied from 2,400 to 13,120. Of these cells, the majority was lymphocytes. Davis and Carlson[1] found the number in dogs to vary from 1,000 to 30,000 leucocytes per c. mm., of which from 95 to 100 per cent were lymphocytes.

While it is not certain that the rate of flow of lymph from the thoracic duct through a cannula represents the normal flow into the jugular vein, especially since the animal is anesthetized,[3] yet, when such flow remains fairly constant over a period of hours, one must assume that it approximates the normal flow. If, with this point in mind, we calculate, from fourteen dogs in Rous'[2] series on a basis of the lymphocyte content of the thoracic duct per cubic millimeter and the rate of flow during fixed intervals of his experiments, we find a surprisingly large number, 3,300,000,000 cells, as the total number that

[1] Davis, B. F., and Carlson, A. J., *Am. J. Physiol.*, 1909–10, xxv, 173.

[2] Rous, P., *J. Exp. Med.*, 1908, x, 238, 329, 537.

[3] All operations were performed under ether anesthesia.

would enter the blood stream during 24 hours. This is a larger number than that of lymphocytes present in the blood stream at any one time. Davis and Carlson agree with this estimate in their statement that "many more lymphocytes enter the blood with the lymph in the course of 24 hours than can be found in the blood at any given time."

Given these findings, then, of the entrance of an enormous number of lymphocytes into the blood each day, and the further findings of a relatively constant count of such cells in the circulating blood, a problem of great interest is presented as to the fate of these cells. As many cells must disappear from the circulation each day as are introduced into it, or we would find not a constant count but an ever increasing accumulation of these cells in the blood stream. It is with this problem that the present paper is concerned. There would seem at the outset only three possibilities as to the fate of the lymphocyte: (1) It might be changed in development into some other type of leucocyte. (2) It might disintegrate in the blood stream. (3) It might leave the blood stream. Since the first possibility seems remote, as a result of modern work on hematology, we think that it may be dismissed from consideration and have therefore confined our work to the two other possibilities.

EXPERIMENTAL.

Our work has been done on rabbits, and although chiefly the fate of the lymphocyte has been studied, a few observations have been made on these cells in the thoracic duct and on the rate of flow. Direct counts, obtained not on freely flowing fluid, but from fluid from the duct immediately after the death of the animal, showed from 20,000 to 50,000 cells per c. mm. in four animals, with a proportion of 80 per cent of small lymphocytes to 20 per cent of larger lymphoid cells in counts of 1,000 cells. Granular polymorphonuclear cells and red cells do not appear as normal constituents of the thoracic duct lymph of the rabbit. Agglutinated masses of poorly staining lymphocytes, apparently disintegrating, are quite numerous in stained films of duct lymph. The possible significance of these will be referred to later. We have, in one of two attempts, succeeded in catheterizing the thoracic duct in the rabbit and we obtained lymph flow at the rate of 1 cc. in 10 minutes, in an etherized animal. This does not

necessarily represent the normal rate of flow. The animal is in an abnormal position; the rate and depth of its respiration are abnormal and seem to determine the progress of the column of fluid through the cannula. As the cannula must be of almost capillary size, capillary attraction must be a factor in rate of delivery; on the other hand, the viscosity of the lymph prevents free dropping from the end of the cannula. Withal, it may be assumed that a very considerable number (a billion or more) of lymphocytes enter the blood from the thoracic duct in the course of a day.

Determination of the Rapidity of the Disappearance of Lymphocytes from the Blood.

In a series of experiments on the rabbit, we attempted to cut off the inflow of lymphocytes into the circulation and thus to determine the rapidity of their disappearance from the blood. As a primary procedure, animals were splenectomized under ether anesthesia to exclude the direct entrance into the blood of lymphocytes from the Malpighian corpuscles. Then, after a sufficient interval for recovery and for return of the leucocyte formula to approximately normal, the thoracic duct was occluded by a ligature which included both the thoracic duct and the jugular vein just below the entrance of the former into the jugular. In later experiments the neck lymphatic trunks on the right and left sides were also tied. Since the results of these experiments were uniform, as far as the trend of the change in the lymphocyte count is concerned, although there was a variation in degree, it does not seem necessary to report the series in detail, especially as the procedure is not new and the results do not differ from those of others. The two protocols below illustrate the results.

Rabbit 1.—Male; weight 1,500 gm. December 1, 1920. Spleen removed under ether and with aseptic precautions. Recovery, apparently without complications. December 9. With careful surgical technique one ligature was placed around thoracic duct and left jugular, and a second about the main right cervical lymph trunk. Blood counts, made from the right ear with free flow of blood, are given in Table I.

In this animal there is a drop in lymphocytes of 1,826 per c. mm. in 5 hours and 1,955 in 10 hours after partially cutting off the supply,

with a gradual return toward the normal number. On the basis of 75 cc. (5 per cent of body weight) of blood this primary drop represents the disappearance from the blood stream of 136,950,000 lymphocytes in 5 hours. The simultaneous drop in polymorphonuclears is definitely due to migration into the operative field.

TABLE I.

Time.	Total No. of leucocytes.	Lymphocytes.		Neutrophils.		Basophils.		Large mononuclears.	
		Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.	No.
Before splenectomy.....	8,000	28	2,240	61	4,880	6	480	5	400
" tying thoracic duct.	8,500	34	2,890	60.5	5,142	2.5	212	3	255
5 hrs. later.....	5,600	19	1,064	70	3,920	3	168	8	448
10 " "	5,500	17	935	66	3,630	4	220	13	715
24 " "	8,000	24	1,920	58	4,640	5	400	13	1,140
2 days "	7,600	23	1,748	59	4,484	4	304	14	1,064
3 " "	6,900	22	1,516	61	4,209	4	276	13	897
4 " "	8,300	22	1,826	60	4,980	5	415	13	1,079
6 " "	8,100	25	2,116	58	4,698	4	324	12	972

TABLE II.

Time.	Total No. of leucocytes.	Lymphocytes.		Neutrophils.		Basophils.		Large mononuclears.	
		Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.	No.
Before splenectomy.....	4,100	51	2,091	41	1,678	3	123	5	205
" tying thoracic duct.	9,000	53	4,770	36	3,240	5	450	6	540
6 hrs. later.....	4,500	8	340	85	3,825	4	180	3	135
12 " "	6,200	5	310	86	5,332	5	310	4	248
24 " "	6,700	15	1,005	73	4,891	5	335	7	469
48 " "	12,000	11	1,320	78	9,160	2	240	9	1,080
4 days "	9,700	13	1,251	72	6,984	3	291	12	1,164
6 " "	7,600	25	1,900	66	5,016	3	228	6	456
10 " "	7,700	40.5	3,118	50.5	3,888	3.5	277	5.5	423

Rabbit 2.—Male; 2,000 gm. December 7, 1920. Splenectomy. Recovery took place without apparent complications and none was found at subsequent autopsy. January 5, 1921. The thoracic duct and jugular were included in a ligature; a second was placed above to include the left cervical trunk; the right cervical main trunk was tied, and an attempt was made to dissect free the lymphatic trunks in the neighborhood of the right jugular and subclavian junction. The tissues were closed in layers by interrupted sutures. The blood counts in this animal are given in Table II.

In this animal, with probably better exclusion of lymphocytes, there is a drop in the number of lymphocytes of 4,430 per c. mm. in 6 hours, or, on a basis of 100 cc. of blood, a total disappearance from the circulation of 443,000,000 lymphocytes, a fact well confirmed by the count after 12 hours. This is followed, as in the previous animal, by a gradual return toward normal.

In this last animal the most successful exclusion of lymphocytes was obtained. Success depends upon the character of the main lymph trunks. The rabbit is not satisfactory for such experiments since there is frequently a right thoracic duct as well as a left, as pointed out by Gage[4] and as our injections of experimental animals, post mortem, have indicated. There are also many anastomosing lymph vessels near the entrance of the duct into the jugular. Injection of the thoracic duct through the capsule of the mesenteric lymph glands in one of our animals showed anastomosing vessels leading through the thymus toward the right side. It is through the utilization of such channels that the lymphocyte count in the blood is gradually restored.

Fate of the Lymphocyte.

With these indications of a large supply of lymphocytes and, as is consistent with this, a rapid disappearance of these cells when the supply is cut off, the question as to their fate is still left unanswered. Do they disintegrate in the blood stream or do they leave it? Occasional disintegrating lymphocytes are found in stained blood films made from normal and from diseased individuals, and it seems unquestionable that some lymphocytes may go to pieces in the blood. In an attempt to answer the question as to whether this is the fate of the large majority of lymphocytes, an experiment was performed, which, though not leaving them in a strictly physiological state, simulated this condition as closely as possible. After the number of lymphocytes in the freely flowing blood from the marginal vein of the right ear had been established, a section of the right jugular vein full of blood was isolated between carefully applied double ligatures. Counts of the cells in this section were made 6 hours later in some

[4] Gage, S. H., in Buck, A. H., Reference hand-book of the medical sciences, New York, 2nd edition, 1902, v, 624.

animals and 24 hours later in others. These experiments are open to objection since the cells are probably exposed to a reduced oxygen tension in the stagnant blood and the varied ameboid motion of cells and the settling of corpuscles cause an uneven distribution at the end of 24 hours which makes the counts somewhat questionable. Yet, as they have a bearing on the length of life of the lymphocyte, three counts are given (Table III).

TABLE III.

Rabbit No.	Site of count.	Total count.	Lymphocytes.		Neutrophils.		Basophils.		Large mononuclears.	
			Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.	No.
3	Count from right ear.	7,800	41	3,198	52	4,056	5	390	2	156
	" " jugular section 6 hrs. after ligation.	7,250	63	4,567	30	2,175	5.3	384	16	116
4	Count from right ear.	7,250	52	3,770	37	2,682	8	580	4	295
	" " jugular section 24 hrs. after ligation.	4,600	62	2,852	32	1,372	4	184	2	92
5	Count from right ear.	9,000	36	3,240	50	4,500	2	180	13	170
	" " jugular section 24 hrs. after ligation.	6,800	53	3,604	40	2,720	2	136	5	340

In spite of the inaccuracies of the method, there is apparently a greater disappearance of polymorphonuclear cells than of lymphocytes. This was explained by histological sections made through the vein wall, which showed not that these cells were destroyed, but that there was a marked migration of them toward the operative field. In the stained film from the jugular blood, the most striking feature is the fact that the lymphocytes show no tendency toward disintegration but are as sharply stained and clear-cut in the differentiation of nucleus and protoplasmic rim as in films from the freely circulating blood. We believe that the experiments, though faulty, indicate that there is not the rapid disintegration of the cells which would be necessary to explain the rate of their disappearance from the blood when the supply is cut off. They must leave the blood stream.

Since the ameboid motion of lymphocytes is generally accepted, there is no objection *a priori* to the proposition that they leave the blood stream under normal conditions in the numbers demanded by the figures given. The question arises as to where they go. The small accumulations of lymphocytes found in the organs generally will not account for the vast number that leaves the blood vessels, unless the cells are destroyed in the tissues as rapidly as we postulated their destruction in the blood stream, and of this there is no evidence in normal organs. Without destruction, of necessity there would be an increasing accumulation in these organs such as may be seen in lymphocytic leucemias.

A search of the organs in histological section leads to the conclusion that the great majority of these lymphocytes enter the mucosa of the gastrointestinal tract and, in addition, pass through it into the intestinal lumen. In sections of rabbit intestine removed immediately after the death of the animal and fixed rapidly in formaldehyde without opening or disturbing the contents, the number of cells in the mucosa, between the cells of the intact epithelial lining, and in the intestinal content is surprising, not only in the parts of the tract where lymphocyte production takes place, as in the lower ileum and the appendix, but also high in the duodenum. If the 40 or 50 lymphocytes which may be counted between the epithelial cells of the tip of a duodenal villus in a 10 micron section are multiplied by its probable relation to the surface area of the intestine, there is no difficulty in accounting for all the lymphocytes that disappear from the circulation. Where there is local production, as in the lymphoid nodules of the appendix, several hundred lymphocytes and large mononuclears may be found in a section through the mucus of a single crypt. In a section of the intestine made without disturbing the contents of the lumen, collections of lymphocytes and larger mononuclear cells are numerous close to the mucosa, especially in the small intestine, even, as stated above, high in the duodenum. In the rabbit colon, which contains inspissated fecal masses, there is little or almost no migration of lymphocytes. The scarcity of polymorphonuclear leucocytes throughout the intestine is as striking as the presence of the mononuclear cells.

Several attempts were made to count the free lymphocytes in the intestinal content by taking up into a calibrated capillary tube a

known amount of fluid close to the mucosa, spreading it over a small part of a cover-glass, staining, and counting the total number of cells. Such a count gave 100 definite lymphocytes per c. mm. for the upper duodenum, 480 for the ileum, and 700 for the appendix. These figures are without significance when taken in relation to the total volume of the intestinal contents, as the lymphocytes are not evenly distributed throughout the contents, at least in recognizable form, but lie close to the mucosa. However, they indicate that large numbers of lymphocytes enter the lumen normally. In fact, one may speak of a normal excretion of lymphocytes from the intestinal mucosa. This takes place not only from the blood stream but from the lymphoid nodules and patches, which are shown by sections to be delivering lymphocytes to the intestine on one side, and to the efferent lymphatics on the other. The latter cells must take an indirect route through the thoracic duct and the blood stream to reach the lumen. To a slight degree the same process is visible in the bronchial mucosa of the rabbit.

It would seem then that under normal conditions the lymphocyte fulfills its function to a large degree upon the surface of the intestinal mucosa. To attempt to designate that function would, with our present knowledge, be a matter of almost pure speculation. In attacking the problem, it is difficult not to be influenced by the immunity of the intestinal mucosa to the countless bacteria within its lumen and to their toxins. The lymphocyte is not phagocytic, but there are many indications that it may affix toxins, and among these may be cited the agglutination of lymphocytes noted in the thoracic duct.

CONCLUSION.

Although the count of circulating lymphocytes in the blood stream remains constant, more lymphocytes enter the blood from the thoracic duct during 24 hours than are present in the blood at any one time. This excess of lymphocytes is not destroyed in the blood stream. The cells migrate from the blood vessels into the mucous membranes and through them to their surface. This occurs chiefly in the gastrointestinal tract, and it is apparently in the mucosa and especially within the intestinal lumen that the function of the lymphocyte is normally performed.

RELATION OF ANTIBODY AND ANTIGEN TO SERUM DISEASE SUSCEPTIBILITY.

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Longcope and Rackemann[1] have reported observations on the precipitin, anaphylactic antibody, and cutaneous hypersensitiveness in individuals to whom serum had been administered therapeutically. In the course of these studies, two patients were encountered who developed no serum disease. It was found that in the blood of these patients, no precipitin was demonstrable. The studies reported in the present paper were undertaken with a threefold purpose: (1) to pursue further the investigation of the relations between precipitin formation and the symptoms of serum reactions; (2) to determine whether or not the disappearance of horse serum from the circulation can be brought into relation with precipitin formation or the symptoms; and (3) to investigate further the factors concerned in the non-susceptibility of certain individuals to serum sickness.

Method.

The presence in the patients' serum of the antipneumococcus or antimeningococcus horse serum, which we refer to as precipitinogen, was determined by specific precipitation with the serum of rabbits immunized against horse serum. This anti-horse rabbit serum was used in all the tests without inactivation, and without preservatives, but was diluted with an equal volume of normal salt solution. On each day on which tests were done, the rabbit serum was titrated

* The participation of Dr. Leake in the investigation reported in this paper was made possible by the donation of a fund by Mrs. Arthur W. Swan.

[1] Longcope, W. T., and Rackemann, F. M., *J. Exp. Med.*, 1918, xxvii, 341.

against dilutions of normal horse serum, and to insure the specificity of the reactions, it was titrated against several dilutions of normal human serum. Owing to the frequent occurrence of prozone phenomena, it was found necessary to set up several dilutions of each of these controls, because sometimes an anti-horse rabbit serum which gives no precipitation with human serum in dilutions of 1:2 or 1:10 will give a definite clouding or actual precipitate in dilutions of 1:100 or 1:1,000. Obviously, any such undetected non-specific reactions would lead to false conclusions as to the presence of precipitinogen in the patients' serum.

In the determination of precipitinogen, there is another possible source of error which must be guarded against. It is, of course, well known that an immune serum may contain both precipitin and precipitinogen without forming a precipitate.[2, 3] But if two such sera are mixed, precipitation follows. In determining the titer, therefore, of the precipitinogen in the patient's serum, it is important to use only immune sera which have been tested and found to be free from precipitinogen. Otherwise when precipitin is present in the serum of the patient, it will precipitate these persisting traces of precipitinogen in the immune rabbit serum and make it appear that precipitinogen is still present in the circulation of the patient. The absence of precipitinogen from the immune rabbit serum is demonstrated by securing two anti-horse rabbit sera which, when titrated against each other, produce no precipitation.

Determinations of the precipitin in the patient's serum were made by the usual method of setting up the patient's serum, diluted with an equal volume of salt solution, against several dilutions of horse serum. In all the tests, both for precipitin and precipitinogen, a constant amount of the precipitating serum was added to increasing dilutions of precipitinogen.

Usually the tests were done on the same day that the blood was drawn, but in a few instances the serum was kept in the ice box over night before being used. After being set up, the tubes were placed in the water bath for 1 hour at 37°C. and then in the ice box over night, when the final reading was made.

[2] Weil, R., *Proc. Soc. Exp. Biol. and Med.*, 1914–15, xii, 37.

[3] Denzer, B. S., *J. Infect. Dis.*, 1916, xviii, 631.

The delicacy of these tests is considerably increased if the serum is perfectly clear. For several hours after an ordinary meal, the serum is so clouded with fat that accurate readings are impossible. Whenever possible, therefore, the blood was drawn after a 14 hour fast. This almost always insures a perfectly clear serum.

Material.

By the methods described above, we have studied twenty-one patients to whom serum was administered for therapeutic purposes either intravenously or intraspinally in amounts varying from 34 to 630 cc. There were, however, two patients in this group who, we felt, should be excluded from analysis because of complicating factors, affecting to an unknown degree the phenomena under consideration. One of these was a patient with lobar pneumonia, Type I, syphilis, and a severe amebic colitis. As he was having frequent watery stools, it seemed probable that he might be eliminating the foreign serum through the intestinal mucosa. If such were the case, the precipitinogen would disappear from the circulation earlier than it would if only the usual mechanism for elimination were operating. The same consideration holds for the other patient excluded from analysis. This patient was a child 5 years old with Pneumococcus Type I peritonitis and empyema. Laparotomy and thoracotomy were done and the abdomen and pleural cavity were drained. It was therefore thought to be highly probable that she was eliminating precipitinogen through the abdominal and thoracic sinuses and that conditions were not comparable to those existing in the other patients. It is noteworthy that the discrepancy observed in these two patients in the interrelations of precipitin, precipitinogen, and symptoms was merely the unusually early disappearance of precipitinogen from the circulation.

The nineteen patients remaining for consideration varied in age from 13 to 56. There were thirteen males and six females. They were treated with antipneumococcus or antimeningococcus serum in amounts varying from 34 to 630 cc. Eighteen were patients with lobar pneumonia, Type I, and one was a patient with meningococcus meningitis. Only one of the patients showed cutaneous hypersen-

sitiveness prior to the therapeutic administration of serum. The single individual who was hypersensitive when he came under observation will be referred to again. These data regarding the patients studied are shown in Table I.

TABLE I.

Patient No.	Age.	Sex.	Diagnosis.	Amount of serum.	Method of administration.	Intracutaneous test with horse serum before administration of serum.
	yrs.			*cc.*		
1	56	M.	Lobar pneumonia.	100	Intravenously.	Negative.
2	20	"	" "	200	"	"
3	39	F.	Meningococcus meningitis.	95	Intraspinally.	"
4	25	M.	Lobar pneumonia.	500	Intravenously.	"
5	25	F.	" "	300	"	"
6	24	"	" "	320	"	"
7	32	M.	" "	480	"	"
8	20	"	" "	300	"	"
9	37	"	" "	630	"	"
10	25	"	" "	370	"	"
11	32	F.	" "	270	"	"
12	22	M.	" "	34	Subcutaneously, 2 cc. Intravenously, 32 "	Positive.
13	23	F.	" "	500	"	Negative.
14	13	M.	" "	180	"	"
15	25	"	" "	380	"	"
16	19	"	" "	385	"	"
17	28	"	" "	520	"	"
18	26	"	" "	200	"	"
19	32	F.	" "	500	"	"

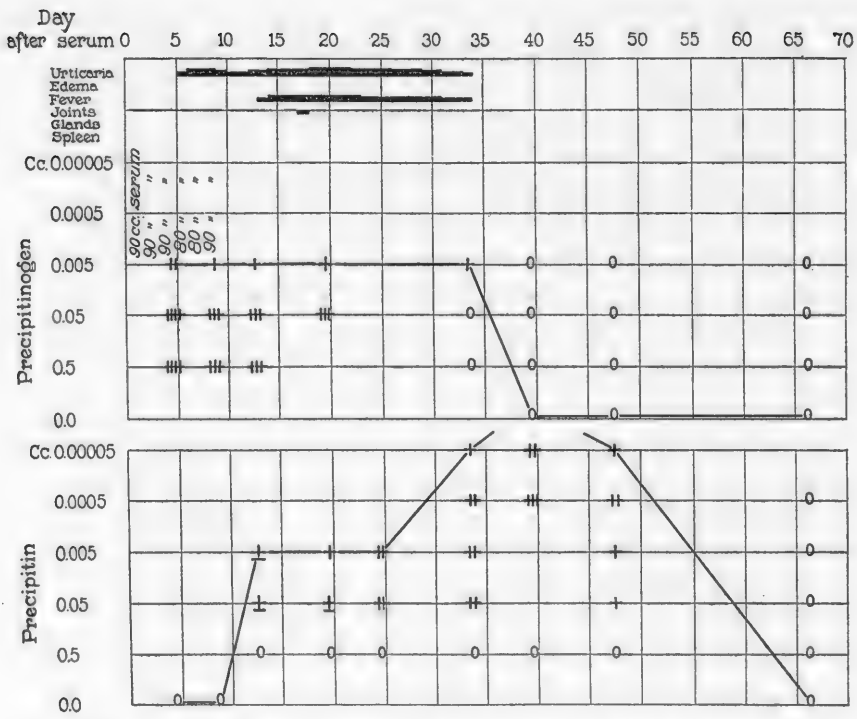
RESULTS.

Analysis of the results obtained in this series of nineteen patients shows that from the point of view of circulating precipitin, persistence of precipitinogen, and severity of symptoms, they may be divided into three groups.

Group 1.—Eleven of the nineteen patients fall quite sharply into one group. This group has the following characteristics: (1) Severe serum disease. If the onset of the serum reaction is considered to be marked by the appearance of an eruption, edema, or arthralgia, and

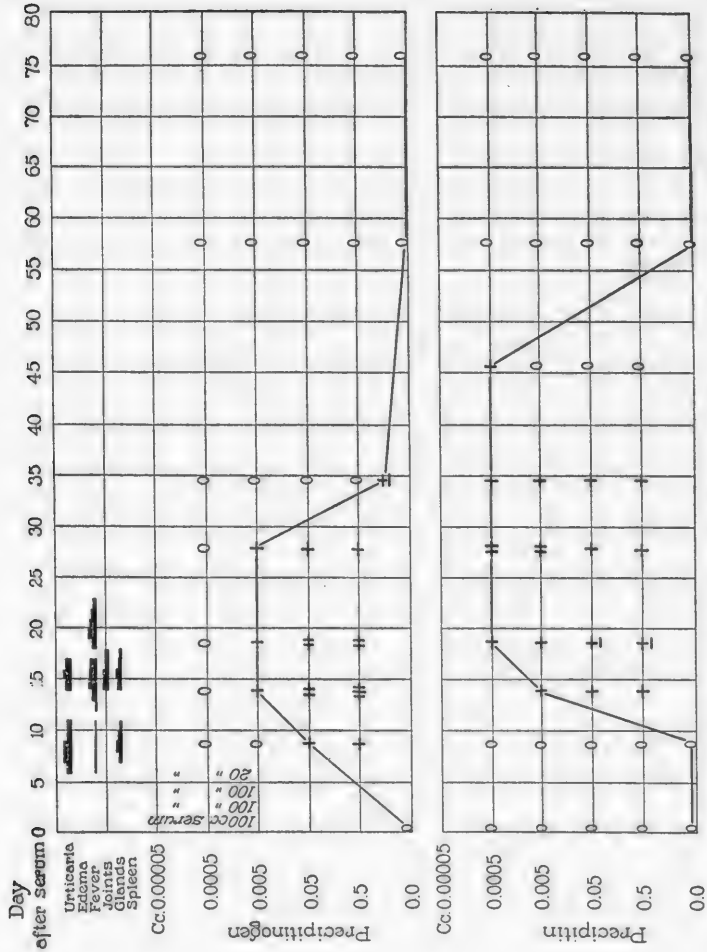
the termination of the serum reaction to be marked by the disappearance of these symptoms, ten of the eleven patients in this group had serum disease lasting 7 days or longer. The other patient had an urticarial eruption lasting 6 days. (2) A relatively high titer of precipitin appearing first or rising to the crest of the curve near the time that symptoms are subsiding. In only one of the patients in this group was the precipitin titer at the height of the curve less than 1:2,000. (3) Disappearance of precipitinogen from the circulation near the termination of the symptoms of serum disease. The usual form of curve for precipitinogen was a steady persistence at a high level until the precipitin curve rose sharply to its high point; a steep decline in the precipitinogen curve then occurred. Efforts were repeatedly made to demonstrate the presence of the foreign serum in the urine, but these tests were, without exception, negative. It seems probable, therefore, that the circulating precipitinogen is not eliminated by the kidneys, at least not in a form demonstrable by specific precipitation.

The characteristic interrelations of the three factors under consideration—precipitin, precipitinogen, and symptoms—are shown in Text-figs. 1 to 4. In Text-fig. 1, it is seen that the serum reaction began on the 5th day and persisted until the 34th day, that precipitin first appeared in the circulation on the 12th day, and rose to the crest of the curve synchronous with the disappearance of horse serum from the circulation and near the time that the symptoms subsided. Text-figs. 2 and 3 show very similar relations between precipitin, precipitinogen, and symptoms. Text-fig. 4 also conforms to this type but is of special interest because this patient had twice previously been treated with horse serum; 7 years before, he had been given diphtheria antitoxin without any reaction, and 2 years previously, he had meningococcus meningitis and was given antimeningococcus serum intraspinally. About 2 weeks later, he developed a severe attack of urticaria and his eyes were swollen. When we first saw him, he gave a positive intracutaneous reaction to horse serum and an attempt to desensitize him by the Besredka method of fractional doses was attempted. After a total of 34 cc. had been given, he developed an immediate reaction consisting of erythema and urticaria. This persisted for 24 hours, then 2 days later, that is on



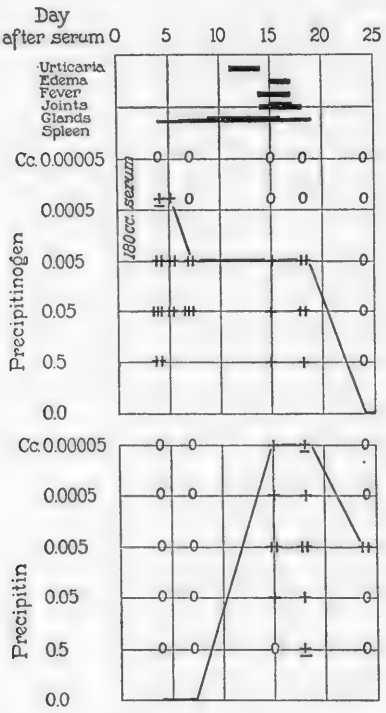
TEXT-FIG. 1. Patient 17 (Table I). Lobar pneumonia, Type I. Serum injections were made on the first 4 days.

In all the text-figures the abscissæ represent the time in days following the first administration of serum. The ordinates represent the dilutions of antigen used in the tests for precipitin and precipitinogen. At the top of the figure the symptoms of the serum reaction are shown in black. The results of the precipitin reactions in the various dilutions employed are shown by + and 0 signs. In the text-figures in which the amounts of serum for the different injections are shown it has sometimes been necessary, in order to make the lettering large enough to be legible, to include a larger number of days for serum injections than was actually involved. When this has been done the actual number of days on which serum was given, is indicated in the legend of the figure.



TEXT-FIG. 2. Patient 6 (Table I). Lobar pneumonia Type I. Serum injections were made on the first 2 days.

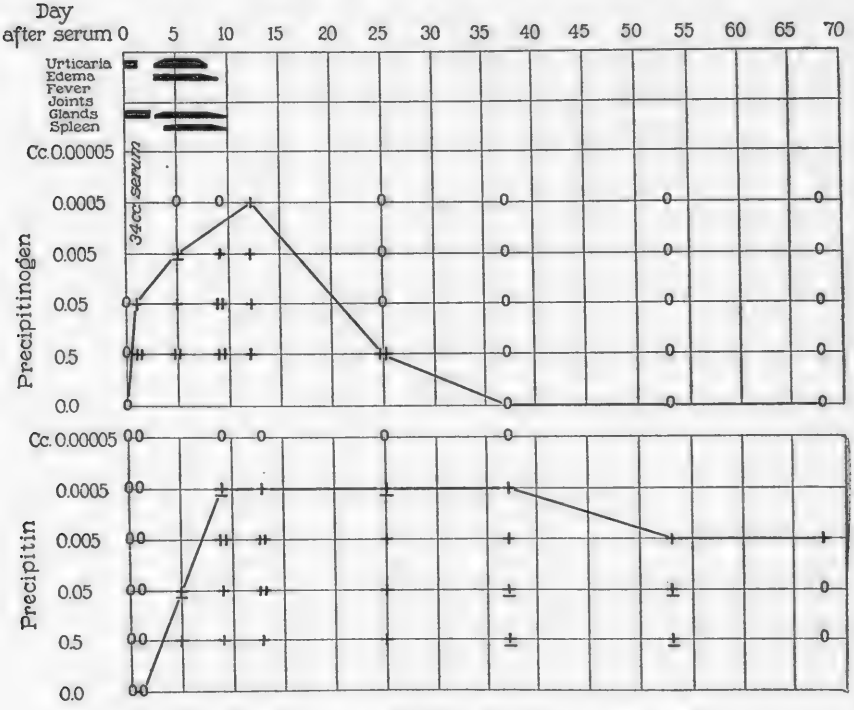
the 3rd day following the serum injections, an accelerated reaction developed, characterized by a morbilliform, urticarial, and purpuric eruption, edema, and enlargement of the lymph nodes and spleen. The particularly interesting feature was the accelerated appearance of precipitin, parallel with the accelerated symptomatic reaction. It is readily seen in Text-fig. 4 that the interrelations between pre-



TEXT-FIG. 3. Patient 14 (Table I). Lobar pneumonia Type I.

cipitin, precipitinogen, and symptoms were essentially the same in this reinjected patient, who showed immediate and accelerated reactions, as in patients receiving serum for the first time. The chief difference appears to be a shortening of the incubation period both for symptoms and for antibody formation. This is comparable to the early appearance of antibodies upon reinjection of rabbits pre-

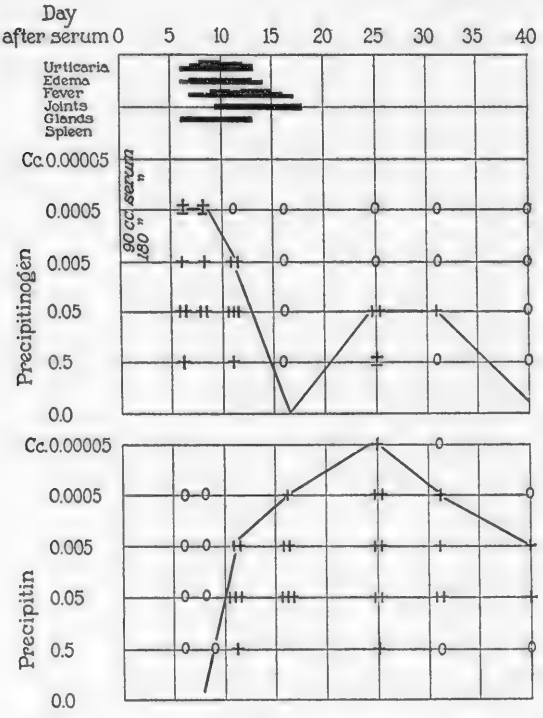
viously sensitized, but without antibody free in the circulation at the time of reinjection. The other seven patients in this group show similar relations between precipitin, precipitinogen, and symptoms. No two are exactly alike, nor would one, from analogy with antibody formation in animals, expect them to be so. The duration of the symptoms, the rapidity with which the precipitinogen disappears,



TEXT-FIG. 4. Patient 12 (Table I). Lobar pneumonia.

and the height of the precipitin curve show considerable variations, but in an unmistakable way, the characteristics of this group are manifest in all of the eleven patients. However, there are two patients in this group presenting a phenomenon demanding further comment. In Text-fig. 5, it is seen that following the rise of precipitin on the 11th day, the precipitinogen completely disappears from

the circulation, but appears again later, and after 9 days gives a positive reaction in dilutions up to 1:200. In following precipitin and precipitinogen in the circulation of rabbits, we have several times encountered a similar phenomenon. It seems as if under certain conditions the union of precipitin and precipitinogen were

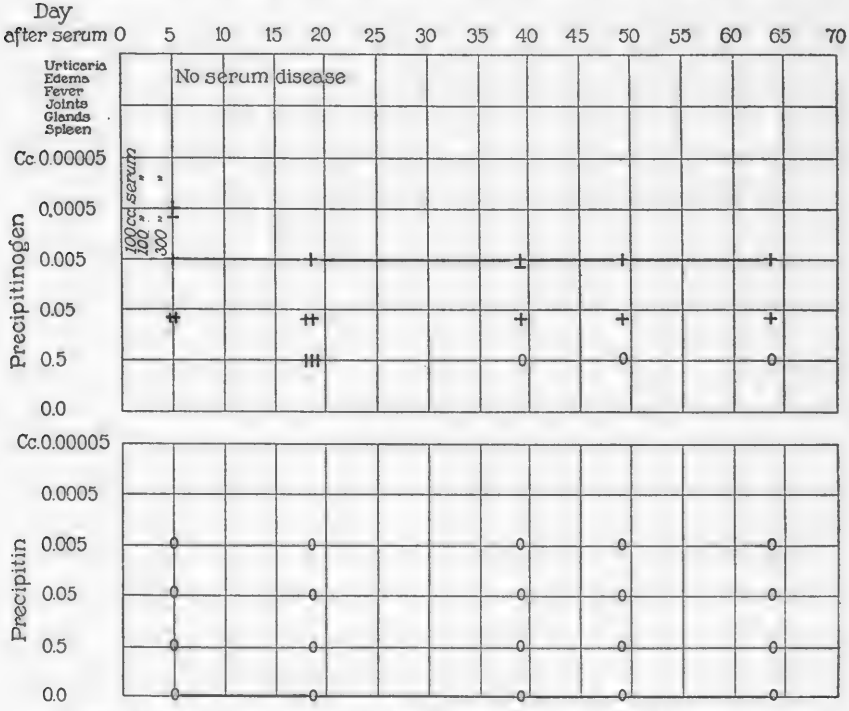


TEXT-FIG. 5. Patient 11 (Table I). Lobar pneumonia Type I. Serum injections were made on the first 2 days.

unstable and after being bound for a time by precipitin, the precipitinogen is subsequently released and circulates freely in the blood stream. We have no adequate explanation to offer for this reappearance of precipitinogen.

Group 2.—The patients in this group, four in number, form an interesting contrast with those just described. Instead of a severe

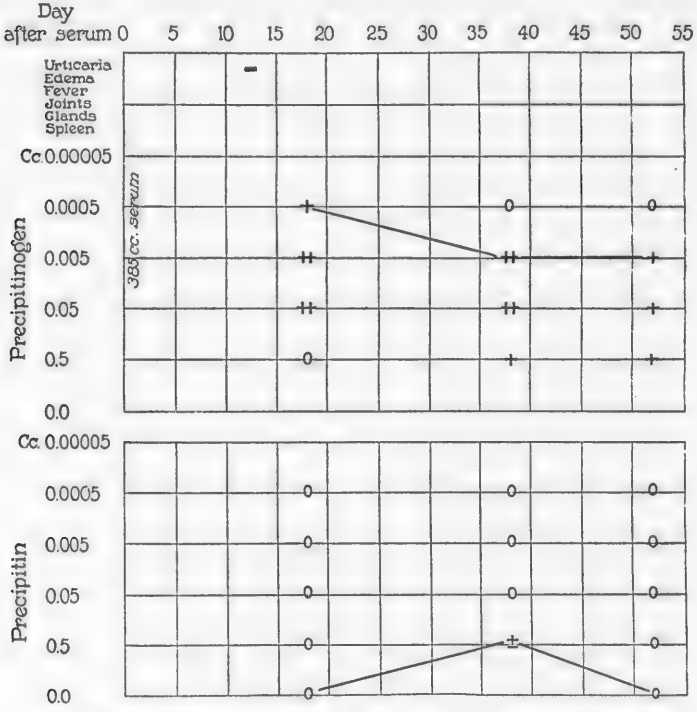
serum reaction, there is none at all or only very mild symptoms; instead of a high titer of precipitin, none is demonstrable in the circulation, or perhaps traces are present for a short period; instead of an early disappearance of precipitinogen, in each of the four patients of this type, there was a positive reaction for precipitinogen as long as the patient could be kept under observation—from 52 to 67 days.



TEXT-FIG. 6. Patient 13 (Table I). Lobar pneumonia Type I. Serum injections were made on the 1st, 2nd, and 4th days.

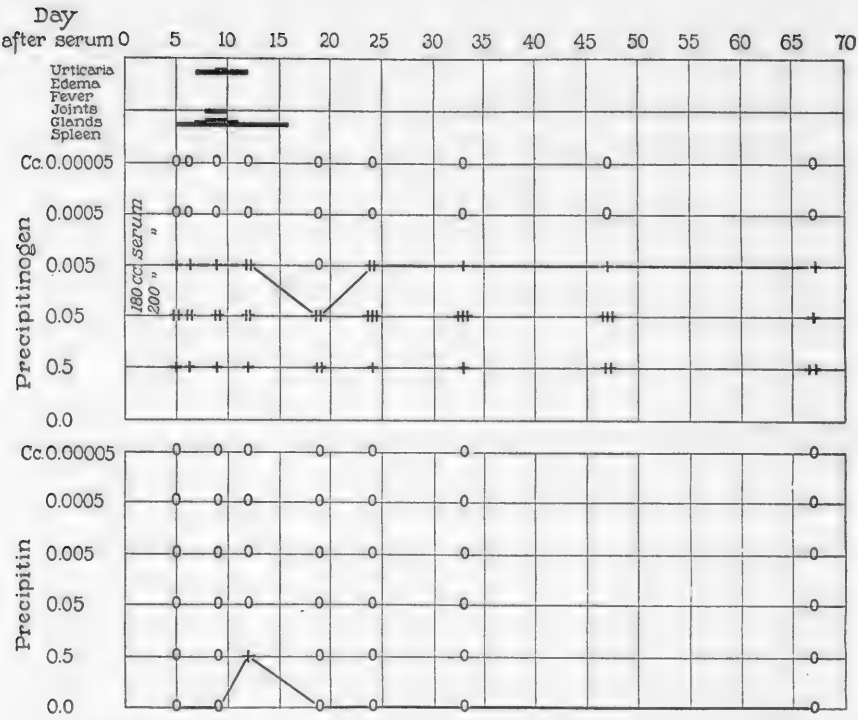
Text-figs. 6 to 8 illustrate the relations between precipitin, precipitinogen, and symptoms which characterize this group. In Text-fig. 6, it is seen that this patient, although given 500 cc. of serum intravenously, had no symptoms of serum disease, that precipitin did not appear in the circulation, and that horse serum was still present in the circulation 63 days after the first administration of serum. Text-

figs. 7 and 8 show relations of the same type between the three factors under consideration, although in each of these patients, there was a mild serum reaction and a transient appearance of precipitin in the lowest dilution.



TEXT-FIG. 7. Patient 16 (Table I). Lobar pneumonia Type I.

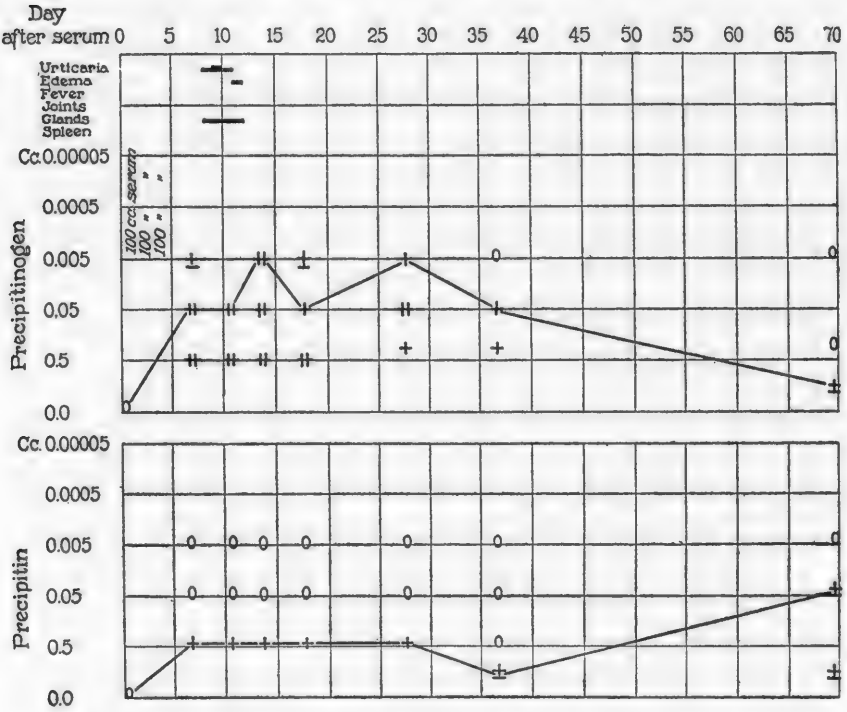
Group 3.—The four patients remaining do not fall definitely into either of the two groups, the characteristics of which have been described. Three of these four patients, however, had mild serum disease and transient or scanty precipitin in the circulation. One of these three is quite clearly a type intermediate between the two groups already described. Text-fig. 9 shows that this patient had a mild serum reaction, a very low titer of precipitin in the circulation, and a curve for precipitinogen which fell slowly, being intermediate



TEXT-FIG. 8. Patient 15 (Table I). Lobar pneumonia Type I. Serum injections were made on the first 2 days.

between the steep decline characteristic of the severe serum disease group and the steady persistence of the precipitinogen characteristic of the group which had no serum disease, or only very slight symptoms. One other patient was perhaps of this same type, but he was lost to observation before the curves could be completed. He had mild serum disease, transient appearance of precipitin, and a precipitinogen curve which showed a decline after the temporary appearance of precipitin, but did not reach the base-line. Owing to the fact that observation of this patient could not be continued, it was not possible to demonstrate that precipitin reappeared in the circulation or to determine when precipitinogen disappeared.

The third patient in this group of four which showed atypical curves was a patient with meningococcus meningitis who was given 95 cc. of antimeningococcus serum intraspinally. She had a very mild serum disease, a low titer of precipitin, and a curve for precipitinogen resembling that found in the patients with severe serum



TEXT-FIG. 9. Patient 5 (Table I). Lobar pneumonia Type I; pregnancy. Serum injections were made on the first 3 days.

disease. It is possible that when serum is administered intraspinally, some additional mechanism comes into play for the disposal of the foreign serum and, therefore, that the interrelations of symptoms, precipitin, and precipitinogen differ somewhat from those found in patients to whom the serum is administered intravenously. Recent

observations by Alexander[4] show that subdural and intravenous injections of the same quantities of foreign serum produce in rabbits distinctly different precipitin curves.

There remains one patient to be considered. This individual was given 630 cc. of antipneumococcus serum intravenously. He had a severe serum reaction; a high titer of precipitin appeared in the circulation synchronous with the cessation of the serum disease, but the precipitinogen, although falling somewhat at the time that precipitin rose to a high concentration, never disappeared entirely from the circulation during the 74 days that he was under observation. This is at variance with the relations which we have found to hold true in the other eighteen patients. There is, however, one consideration which might explain the discrepancy. This patient was one of the first studied, and at this time we had not realized the importance in precipitinogen determination of testing the anti-horse rabbit serum for traces of antigen used in immunizing the rabbit. It is possible, therefore, to explain the discrepancy on the assumption that the precipitating serum used contained traces of antigen and that the precipitin in the patient's serum gave a precipitate with the antigen still present in the anti-horse rabbit serum. This, of course, would make it appear that there was precipitinogen in the circulation of the patient, when it had, perhaps, entirely disappeared. It cannot be proved that this explanation is correct, but it seems plausible.

Summarizing then the results on nineteen patients, we find that eleven of them are distinctly of one type. These are the good precipitin formers who have severe serum disease and get rid of precipitinogen soon after the symptoms have subsided. The second group, contrasting sharply with these, consists of four patients. They are poor precipitin formers, have little or no serum disease, and retain the precipitinogen in the circulation for many weeks and perhaps months. Three of the remaining four patients are more or less distinctly intermediate forms, and the single patient in whom the results appear completely at variance with the observations on the other eighteen may perhaps be explained by a technical error.

[4] Alexander, H. L., *J. Exp. Med.*, 1921, xxxiii, 471.

DISCUSSION.

It has long been known that individuals show wide variations in their reaction to the administration of foreign serum. At one end of the scale is the individual who is naturally hypersensitive to horse serum. He is often a horse asthmatic with an inherited tendency to hypersensitiveness. He is exquisitely hypersensitive, reacting immediately with violent symptoms to even minute quantities of horse serum. Reactions of this type are sometimes fatal. At the other end of the scale is the individual who does not show a serum reaction after the intravenous administration of even several hundred cubic centimeters of serum. There is clearly wide variation in the response to foreign serum among individuals who at the time that serum is first administered are not demonstrably hypersensitive. Some have severe serum disease, some have only mild symptoms of short duration, and some have no symptoms whatever. That such variations, when large amounts of serum are given, are not dependent upon the amount of serum administered is evident from the results in the present series of patients. The average amount of serum given to the four patients in the group showing no serum disease, or only very mild symptoms was 460 cc.; the average amount given to the patients who had severe serum reactions was 270 cc. When smaller amounts of serum are used, as in diphtheria immunization, and the injections are made subcutaneously or intramuscularly, there appears to be a relation between the incidence of serum reactions and the amount used.[5] With the larger doses, the incidence of reactions is higher, but when amounts over 100 cc. are given, the variations in the intensity and duration of the reaction lead to the conclusion that there is an intrinsic difference between the susceptible and the insusceptible individuals.

What we have found to be characteristic of the susceptible group of patients as far as precipitin, precipitinogen, and symptoms are concerned corroborates the observations of Longcope and Rackemann[1] on the relation of circulating precipitin to the cessation of symptoms, and lends further support to the conception of serum dis-

[5] Daut, M., *Jahrb. Kinderheilk.*, 1897, xliv, 289. Sturtevant, M., *Arch. Int. Med.*, 1916, xvii, 176. Weaver, G. H., *Arch. Int. Med.*, 1909, iii, 485.

ease as an antigen-antibody reaction. Efforts to discover the mechanism of antigen-antibody reactions have resulted in the development of two hypotheses which differ as to the site in which the reaction occurs. One school localizes the reaction within the circulation and holds to the view that the reaction is dependent upon an intravascular union of antigen and antibody. The other school believes that this union takes place chiefly within the tissue cells. No attempt will be made to discuss the voluminous and controversial literature on this question, but it suffices to say that, at present, a preponderance of competent opinion supports the cellular hypothesis. With the facts at present available, the most plausible explanation of the mechanism of these reactions[6] is, therefore, that the foreign serum unites with the tissue cells; that as a result of such union, antibodies are produced; that the intracellular union of antigen and antibody causes the symptoms; and that after a time, the antibody is produced in excess and ceases then to be exclusively intracellular. At this time it becomes demonstrable in the circulation. For a time thereafter, there are both antigen and antibody free in the circulation. If large amounts of antibody reach the circulation, the antigen soon disappears. This disappearance of antigen does not necessarily imply an intravascular union of antigen with antibody, although such a mechanism might account for it. It is also possible that at this time, with greater amounts of intracellular antibody available, the tissue cells appropriate the antigen with greater avidity. All that can be stated at present is that during this period when precipitin is abundant, the foreign serum disappears.

The varying degrees of intensity of reaction by susceptible individuals should therefore be thought of either as varying degrees of susceptibility to a toxic product of intracellular antigen-antibody reaction, or as varying degrees of ability to form antibodies. The latter hypothesis seems to accord better with the facts, because, in general, it is the good precipitin formers who have severe serum reactions. The parallelism is not exact, nor would one expect it to be so, unless there were proof that the precipitin in the circulation is exactly proportional to the intracellular precipitin. And there is no

[6] Weil, R., *J. Immunol.*, 1916–17, ii, 399.

proof of this. A possible explanation of such varying degrees of efficiency with which different individuals form antibodies is discussed below.

In the group of patients found to be insusceptible to serum reactions, in whom little or no precipitin is found, and in whose circulation the foreign serum is present for a long period, there clearly is something which acts as a protective mechanism. Three possibilities perhaps offer plausible explanations for the basis of this failure to produce precipitins and the persistence of the precipitinogen in the circulation. (1) In these individuals there may be something in the circulation which stands as a barrier between the foreign protein in the blood stream and the interior of the cells, preventing the union of foreign protein and tissue cell, and thus interfering with an essential phase of antibody production. (2) The tissue cells of these individuals may be impermeable to the foreign serum. If either one of these conditions existed, little or none of the foreign serum would penetrate the cells, and hence little or no antibody would be formed and serum disease would be mild or absent. (3) In terms of Ehrlich's side-chain theory, the difference between the susceptible and the insusceptible individuals might consist in a deficiency or absence of haptophore groups in the tissue cells of the latter. Under any circumstances, with failure of the cells to take up the foreign protein, it might persist in the circulation until slowly disposed of by some other mechanism.

It is interesting in this connection to recall some observations made years ago by Metchnikoff.[7] In studying the mechanism of natural immunity, he found that a number of species were insusceptible to certain bacterial toxins which were extremely poisonous for other species. Spiders and scorpions were found to be unaffected by tetanus toxin. Nor could antitoxin be demonstrated in the blood of these animals, so the natural immunity could not be ascribed to antitoxic properties of the body fluids. In these animals, however, the injected toxin soon disappeared from the circulation. The immunity of fowls to tetanus toxin was known even before Metchnikoff's studies. The fowl also tolerates large doses of tetanus toxin but can nevertheless be tetanized if it is previously weakened by exposure to cold or if sufficiently large doses are used. That this immunity is not due to the presence or production of antitoxin was shown by Vaillard[8] who

[7] Metchnikoff, E., Immunity in infective diseases, translated by Binnie, F. G., Cambridge, 1905.

[8] Vaillard, *Compt. rend. Soc. biol.*, 1891, xliii, 462.

demonstrated that the serum contains no antibody. Moreover, von Behring[9] showed that fowls are highly susceptible if the toxin is injected directly into the brain. Metchnikoff also studied green lizards, marsh turtles, and the larvæ of the rhinoceros beetle (*Oryctes nasicornis*). These animals tolerate, without symptoms, enormous doses of tetanus toxin and not only do not form any antitoxin, but retain the toxin in the circulation for a long period of time, even months.

Phenomena of this type in lower animals suggested to both Metchnikoff[7] and von Behring[10] the possible relation of cell permeability to natural immunity. Our observations on serum disease in man are strikingly analogous to those of Metchnikoff on turtles, lizards, and beetles and suggest anew a factor which may be important in natural immunity.

Investigators in the field of natural immunity have been occupied chiefly in studying different degrees of susceptibility characterizing races or species. On epidemiological grounds alone, it is clear, however, that within any given species or race, there are individual variations in susceptibility to infection for which no adequate explanation exists. From this standpoint, therefore, the demonstration of the differences which we have found to exist between susceptible and insusceptible individuals of the same race may prove to be significant.

SUMMARY.

1. Studies on nineteen patients to whom foreign serum had been administered for therapeutic purposes are reported. Analysis of the results obtained by following the precipitin and precipitinogen in the circulation and comparing these factors with the time of appearance, intensity, and duration of the symptoms shows that the nineteen patients fall into three groups.

2. The first group includes eleven patients. These were good precipitin formers, they had severe serum disease, and the precipitinogen disappeared from the circulation near the time that the symptoms subsided.

[9] von Behring, E., in Eulenberg, A., and Samuel, Lehrbuch der allgemeinen Therapie, Berlin and Vienna, 1899, iii, 992.

[10] von Behring, E., in Eulenberg, A., Encyclopädische Jahrbücher der gesammten Heilkunde, Vienna and Berlin, 1900, ix, 203.

3. The second group includes four patients who had little or no serum disease, in whose circulation little or no precipitin was demonstrable, and in whom the precipitinogen persisted in the circulation as long as the patients could be kept under observation—from 52 to 67 days.

4. The remaining four patients form a more or less distinctly intermediate group.

5. The results lend further support to the conception of serum disease as an antigen-antibody reaction.

6. The possibility that our results indicate a factor which may be important in the mechanism of natural immunity is discussed.

We wish to thank Miss Emily Frühbauer for technical assistance.

STUDIES ON MEASLES.

III. Acquired Immunity Following Experimental Measles.

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It has been shown in a preceding paper[1] that monkeys (*Macacus rhesus*) inoculated intratracheally with unfiltered or filtered nasopharyngeal washings from patients in the prodromal or early eruptive stage of measles exhibit an illness which closely resembles measles in man in its course and symptomatology. It has furthermore been shown[2] that the lesions which develop in the skin and buccal mucous membrane during the course of the infection in monkeys present essentially the same histologic picture that is found in the corresponding lesions of human measles. The experimental infection has been successfully transmitted[1] from monkey to monkey with the development of the same group of symptoms and pathologic lesions in the passage animals. These fundamental points of similarity between measles in man and the experimental disease in monkeys would appear sufficient to warrant the application of the term "experimental measles" to the latter condition. It has, nevertheless, seemed desirable to determine whether further points of resemblance between the two might not be shown.

Since an apparently permanent immunity against reinfection characteristically follows one attack of measles, the same phenomenon should hold true with respect to the experimental disease if the two conditions are to be regarded as similar. Furthermore, an acquired immunity, if present, should theoretically be efficient against a virus of heterologous source as well as against that of homologous origin, since there is little clinical evidence to show that one attack of measles fails to confer an immunity that is effective against all subsequent

[1] Blake, F. G., and Trask, J. D., Jr., *J. Exp. Med.*, 1921, xxxiii, 385.

[2] Blake, F. G., and Trask, J. D., Jr., *J. Exp. Med.*, 1921, xxxiii, 413.

exposures. Authentic reports of repeated attacks of measles in the same individual are so few as to be negligible in this connection. In order to test the validity of the foregoing assumptions a series of reinoculation experiments in monkeys which had recovered from a previous attack of experimental measles has been carried out as described below.

EXPERIMENTAL.

Six monkeys which had previously been inoculated with nasopharyngeal washings from cases of measles and had recovered from the ensuing attack of the experimental disease[3] have been subjected to reinoculation with material containing the virus of measles (Table I). In five instances virus of heterologous source was used, in one the homologous virus. In two monkeys the material, consisting of the supernatant fluid from an 0.85 per cent salt solution emulsion of the skin and buccal mucosa of a monkey killed on the 4th day of experimental measles, was injected intratracheally. In four monkeys whole blood withdrawn from a monkey on the 3rd day of experimental measles was injected intravenously. The intervals elapsing between recovery from the preceding experimental measles and the time of reinoculation varied from 12 to 254 days. None of the six monkeys following reinoculation showed any evidence of infection with the virus of measles, while the control normal monkeys, inoculated at the same time with equivalent amounts of the same material, developed the characteristic symptoms and pathologic lesions of the experimental disease. The protocols follow.

Experiment 1.—June 8, 1920. Monkeys 6, 9, and 19 were injected intratracheally at 12.15 p.m., 12.30 p.m., and 12.45 p.m., respectively, each with 10 cc. of the unfiltered supernatant fluid of an 0.85 per cent salt solution tissue emulsion (Virus MC. 3). The emulsion had been prepared from the minced and ground skin and buccal mucosa of Monkey 16, which was killed on the 4th day of experimental measles, about 24 hours after the first appearance of the exanthem. Monkey 6 had been inoculated Apr. 9, 1920, with pooled, filtered nasopharyngeal washings (Virus AM) from two sisters with measles and had recovered from the ensuing experimental measles on Apr. 23, 46 days before reinoculation. Monkey 9 had been inoculated May 12, 1920, with filtered nasopharyngeal washings (Virus

[3] For a detailed description of the first attack of experimental measles in these animals see Blake and Trask.[1]

MC) from a patient with measles and had recovered from the ensuing attack of experimental measles on May 27, 1920, 12 days before reinoculation. Monkey 19 was a normal monkey and served as a control.

TABLE I.

Immunity Following Experimental Measles.

Monkey No.	First inoculation.			Second inoculation.			
	Date.	Virus.	Result.	Date.	Interval after recovery from first attack.	Virus.	Result.
	1920			*1920*	*days*		
6	Apr. 9	AM	Experimental measles Apr. 16–22.	June 8	46	MC.3*	Remained well.
9	May 12	MC	Experimental measles May 20–26.	" 8	12	" 3	" "
19 (control).				" 8		" 3	Experimental measles, June 14–17. Killed.
2	Mar. 24	RG	Experimental measles Mar. 29–Apr. 4.	Dec. 15	254	JJ. 5	Remained well.
3	" 24	RK	Experimental measles Apr. 3–10.	" 15	248	" 5	" "
5	Apr. 9	AM	Experimental measles Apr. 16–22.	" 15	236	" 5	" "
8	May 12	MC	Experimental measles May 19–27.	" 15	201	" 5	" "
46 (control).				" 15		" 5	Experimental measles Dec. 18–27.

* The figure indicates the number of monkeys through which the virus had been passed.

Monkeys 6 and 9 showed no evidence of measles during 21 days observation. There were no conjunctivitis, no enanthem, and no exanthem, the eyes, buccal mucosa, and skin remaining normal in appearance throughout this period. Monkey 19 after an incubation period of 6 days developed the characteristic symptoms

of the experimental disease. On the 7th day a few discrete hyperemic spots appeared on the labial mucous membranes. On the 8th day the animal was listless; the conjunctivæ were injected; fresh Koplik spots had appeared on the mucous membrane of the cheeks. On the 9th day there was a well developed, confluent enanthem on the mucous membrane of the lips, gums, and cheeks. On the 10th day a few discrete, red maculopapules appeared about the lips, on the chin, and behind the ears. The animal was killed and the infection was successfully transmitted to two other monkeys. Histologic sections of the labial mucosa and tongue show the typical lesions of measles. The endothelial cells of the capillary walls are greatly swollen. There are a marked accumulation of endothelial leucocytes and some serous exudate about the capillaries, especially in the papillæ. In the stratified epithelium of the labial mucosa are many small foci (Koplik spots) showing endothelial leucocytes, serous exudate, and beginning necrosis of the epithelial cells. In some of these the process is more advanced and there is maceration of the epithelium, with shallow ulceration and secondary invasion by polymorphonuclear leucocytes. A few similar foci are seen in the epithelium of the tongue. There is also a more diffuse infiltration of the epithelium by endothelial leucocytes.

Experiment 2.—Dec. 15, 1920. Monkeys 2, 3, 5, 8, and 46 were injected intravenously in turn, each with 5 cc. of citrated whole blood (Virus JJ. 5) withdrawn from Monkey 36 on the 3rd day of experimental measles about 6 hours after the first appearance of the exanthem.

Monkey 2 had been inoculated Mar. 24, 1920, with nasopharyngeal washings (Virus RG) from a patient with measles and had recovered from the ensuing attack of experimental measles on Apr. 5, 254 days before reinoculation.

Monkey 3 had been inoculated Mar. 24, 1920, with nasopharyngeal washings (Virus RK) from a patient with measles and had recovered from the ensuing attack of experimental measles on Apr. 11, 248 days before reinoculation.

Monkey 5 had been inoculated Apr. 9, 1920, with pooled nasopharyngeal washings (Virus AM) from two cases of measles and had recovered from the ensuing attack of experimental measles on Apr. 23, 236 days before reinoculation.

Monkey 8 had been inoculated May 12, 1920, with nasopharyngeal washings (Virus MC) from a case of measles and had recovered from the ensuing attack of experimental measles on May 28, 201 days before reinoculation.

Monkey 46, normal, served as a control.

Monkeys 2, 3, 5, and 8 showed no evidence of infection during 21 days observation. They were well and active throughout this period. There were no conjunctivitis, no enanthem, and no exanthem. Monkey 46, after an incubation period of 3 days, developed the characteristic symptoms of measles. On the 4th day three Koplik spots appeared on the mucous membrane of the upper lip. On the 5th day the conjunctivæ were injected. On the 6th day a cluster of fresh Koplik spots was present on the mucous membrane of the lower lip. The animal was drowsy and listless. On the 7th day there were confluent patches of hyperemic enanthem studded with minute white specks on the labial mucosa. A few

red maculopapules appeared on the lower abdomen and inner surfaces of the thighs. By the 10th day there was a thick, red, maculopapular exanthem on the face, neck, chest, abdomen, and legs. By the 12th day the enanthem had faded; the exanthem was fading and showed fine branny desquamation. By the 14th day the animal had recovered except for slight remaining desquamation. Blood cultures on the 5th, 6th, and 7th days showed no growth. The infection was successfully transmitted from this animal to another monkey by means of blood withdrawn on the 5th, 6th, and 7th days. A section of skin excised[4] from the thigh on the 10th day shows the characteristic histologic picture of measles. About the capillaries and small veins in the upper layers of the corium there is a marked accumulation of endothelial leucocytes. Occasionally one is seen in mitosis. A few polymorphonuclear leucocytes are also present. Focal accumulations of endothelial leucocytes with vacuolation and necrosis of epithelial cells are seen in the epithelium of many of the hair sheaths and sebaceous glands. The epidermis shows vacuolation and necrosis of the cells of the Malpighian layer in minute foci. These areas are invaded by endothelial leucocytes. In the cornified layer are occasional, small, deeply staining plaques with the remains of minute vesicles beneath them.

DISCUSSION.

The result of the foregoing experiments shows that one attack of experimental measles confers an apparently complete immunity against reinfection with measles for at least a considerable period. In all probability this immunity is permanent. In Experiment 1 it should be noted that Monkeys 6 and 9 were originally inoculated with filtered (Berkfeld N) nasopharyngeal washings. Their subsequent immunity, therefore, not only provides additional evidence of the similarity between human measles and the experimental disease but also tends to confirm the filterable nature of the virus. The strain of virus used in the reinoculation of Monkey 6 was of different origin from the strain with which this animal was originally inoculated, while with Monkey 9 the same strain of virus was employed in both the first and second inoculations. Since there was no apparent difference in the immunity of the two animals it would seem probable that the immunity provided by one attack of experimental measles is as efficient against a heterologous virus as against the homologous one. This is further supported by the result of the second experiment, in which four monkeys originally inoculated with strains of virus from four different sources exhibited a complete immunity against reinfection with a virus obtained from a still different source.

[4] This was done under ether anesthesia.

This result, as has been pointed out, was to be expected. Furthermore, it would suggest the probability that all strains of measles virus are of homologous nature in as far as their property of stimulating immunity is concerned, a fact which, of course, might readily be predicated from clinical observation.

The results of the two experiments, although they do not provide an explanation of the mechanism of acquired immunity against measles, nevertheless suggest certain possibilities. The course of measles, itself, in conjunction with evidence already presented[1] concerning the infectivity of the blood in the experimental disease, leaves little reason to doubt that the virus gains access to the blood by way of the respiratory mucous membrane and is subsequently distributed by the blood stream to the skin and buccal mucosa where it sets up the characteristic lesions of the disease. It is conceivable that the process of immunity against reinfection might reside in the respiratory mucous membrane which, in the immune animal, would present a barrier to invasion of the body by measles virus. While this supposition might serve as a possible explanation of the immunity exhibited by Monkeys 6 and 9 which were reinoculated by the intratracheal route, it is obviously inadequate in the case of the second experiment in which all the monkeys were inoculated intravenously. Since these animals showed an apparently complete immunity, it is clear that the immunity is not solely, if at all, dependent upon a possible barrier offered by the respiratory mucous membrane of the immune animal. That it is a function of the body tissues or fluids would seem more probable. Whether the immunity is humoral or cellular or both, however, only further experiment can determine.

SUMMARY.

It is shown that monkeys which have recovered from experimental measles are immune to reinfection with the virus of measles irrespective of whether the virus is of homologous or heterologous origin. In this respect experimental measles in the monkey corresponds with measles as observed in human beings, and the result is the same whether the virus is inoculated on the respiratory mucous membrane or is injected intravenously.

CONCLUSION.

Experimental measles in the monkey, like measles in man, is followed by an acquired immunity against the disease.

STUDIES ON BLOOD CHANGES IN PNEUMOCOCCUS INFECTIONS.

An Experimental Study of the Formation and Fate of Methemoglobin in the Blood.

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INTRODUCTION.

Methemoglobin Formation in Pneumonia.

The pneumococcus, both *in vitro* (Gilbert and Fournier, 1896; Grüter, 1909; and Peabody, 1913) and *in vivo* (Butterfield and Peabody, 1913), has been shown to transform hemoglobin into methemoglobin. In consequence it seemed that methemoglobin formation might be the chief cause of the cyanosis sometimes observed in pneumonia. A study of the oxygen content and capacity of venous and arterial blood, however (Stadie, 1919), showed that the great and constant abnormality accompanying cyanosis was an increased proportion of reduced hemoglobin to oxyhemoglobin in the arterial blood. Therefore the essential cause of the cyanosis of pneumonia is incomplete oxygenation of the arterial blood, rather than the presence of methemoglobin in the blood. In only one of the thirty-two cases studied by the author was the oxygen capacity significantly reduced, as it would be if any considerable portion of the hemoglobin were changed to methemoglobin. On the contrary, in many cases the oxygen capacity of the blood was above the normal average.

Although we had but one case in which the oxygen capacity fell significantly, Peabody (1913) and Harrop (1919) have observed several cases in which such a fall occurred. Of eleven cases Peabody observed a marked fall of oxygen capacity in three, and Harrop in nine cases found a decrease of one-half in the total oxygen capacity in two. All these cases had positive blood cultures. In two other cases of Harrop's with negative blood cultures there was a decrease in oxygen capacity, but of less degree and over a greater period of time. It is possible that in these cases a considerable proportion of the hemoglobin may be altered into methemoglobin, and even in patients that do not show definitely lowered oxygen capacity small amounts of methemoglobin may be formed, and either be eliminated from the blood, or remain in it. In order to obtain more complete

evidence on these points a quantitative method for determination of methemoglobin was devised (Stadie, 1920), and in the present study it has been utilized in experimental work aimed to obtain evidence on the points in question.

General Conditions for Methemoglobin Formation.

The agents which change hemoglobin to methemoglobin are of varied nature. They include the following groups of known chemical substances: oxidizing substances, ozone, iodine, chlorates, ferricyanides, nitrites, nitrates, and azo compounds; reducing substances, pyrogallol, hydroquinone, hydroxylamine, etc.; organic bases, aniline, phenacetin, acetanilide, and toluidine; salts, sodium chloride in concentration above 1.5 per cent and ammonium sulfate in saturated solution. They also include several races of bacteria: *Streptococcus viridans*, cholera, pneumococcus, Gaertner bacillus, and certain nitrosobacilli (Wallis, 1913–14).

In vitro all these agencies produce methemoglobin with greater or less facility. Potassium ferricyanide rapidly forms methemoglobin from oxyhemoglobin. An amount of oxygen is liberated which is equivalent to the dissociable oxygen originally combined as oxyhemoglobin. Sodium nitrite converts hemoglobin to methemoglobin *in vitro*, but more slowly than potassium ferricyanide. The nitrite liberates an amount of oxygen equivalent to the amount necessary to change the nitrite to nitrate (Barcroft and Müller, 1911-12). In other words, two molecules of nitrite transform one molecule of hemoglobin, producing one molecule of oxygen and one molecule of methemoglobin. Hydroxylamine transforms hemoglobin quantitatively, molecule for molecule (Letsche, 1912).

Of the above agents, we have used in our experiments potassium ferricyanide and sodium nitrite.

Characteristics of Clinical and Experimental Methemoglobinemia.

Agents.—Human cases of methemoglobinemia are the result of poisoning with various agents, of which aniline is perhaps the most familiar. In laboratory animals methemoglobin is produced easily by nitrites, potassium ferricyanide, acetanilide, and the pneumococcus.

Symptoms.—In the overwhelming methemoglobinemia produced experimentally it is difficult to dissociate the symptoms produced by

the drug from those produced by the methemoglobin. Rapid breathing and air-hunger are constant symptoms in severe grades of methemoglobinemia, and when 70 to 80 per cent of the hemoglobin is changed the animal exhibits all the signs of acute suffocation and dies in a short time. In milder instances with a destruction of 25 to 50 per cent of the hemoglobin, usually there are no symptoms in rabbits. It is doubtful whether the mere presence of large amounts of methemoglobin in the body is harmful.

Cyanosis is a regular accompaniment of methemoglobinemia. This, of course, is due to the fact that the presence of methemoglobin in blood gives it a dark color closely resembling that of venous blood. Transformation of as little as 5 per cent of the hemoglobin to methemoglobin in blood gives it a dark color. The blood does not become bright red on exposure to the air and is easily distinguishable from normal oxygenated blood. The cyanosis itself is indistinguishable from the cyanosis due to oxygen unsaturation of the arterial blood. Two important differences, however, may be noted: (1) the cyanosis of oxygen unsaturation usually accompanies pulmonary or heart disease and varies in intensity with change of position, coughing, and exertion; (2) the administration of oxygen may diminish this cyanosis. The final differentiation is made by the spectroscope or by methemoglobin determination, for which a method has been recently published (Stadie, 1920).

Pathology.—No characteristic changes of the organs have been described in animals in which a severe methemoglobinemia has been induced. Certain hemorrhagic changes and areas of necrosis in the liver and spleen have been described, but these have been inconstant and have followed large doses of potassium chlorate, and consequently might easily have been due to this substance rather than to the methemoglobin.

The proportion of hemoglobin changed may be as great as 100 per cent, as after the intravenous injection of sodium nitrite in rabbits. With acetanilide or sodium nitrite in proper doses a reduction in the amount of hemoglobin of 60 to 70 per cent may be brought about easily. The animals often recover; the sudden flooding of the body by so large an amount of methemoglobin is without apparent permanent effect.

Methemoglobin may exist in the blood in two distinct conditions. First, it may be present in the plasma alone, a true methemoglobinemia. This is, however, rare, but is illustrated by Brandenburg's case of potassium chlorate poisoning which showed a rapid decrease of red blood cells from 4,300,000 to 1,600,000 in 6 days. The serum showed methemoglobin spectroscopically. Second, the methemoglobin is present within the red blood cells—a condition of methemoglobincythemia. This is the usual occurrence. Cases of nitrite, acetanilide, and nitrobenzene poisoning and bacterial infections with methemoglobin formers fall into this class.

EXPERIMENTAL.

Fate of Methemoglobin.—Methemoglobin solutions injected intravenously are rapidly eliminated from the blood. Table I shows

TABLE I.

Elimination of Methemoglobin Following Intravenous Injection in Rabbits.

Rabbit No.	Time.	Injection.	Hemoglobin per 100 cc. of blood.	Methemoglobin spectrum.		
				Serum.	Cells.	Urine.
	p.m.		*gm.*			
1	12.10		13.87			
	12.20	2 gm. of methemoglobin intravenously.				
	12.45		12.04	Negative.	Negative.	
	4.00	(Killed.)*	11.87	"	"	++++
	a.m.					
2	9.05		10.03			
	11.00	1.7 gm. of methemoglobin intravenously.				
	11.15		10.03	Negative.	Negative.	++++

* Extracts of lungs, liver, spleen, heart, feces, and intestines showed no methemoglobin spectroscopically.

the extreme rapidity with which methemoglobin dissolved in the plasma disappears. The hemoglobin was determined gasometrically by Van Slyke's method (1918), the methemoglobin by the author's method (1920).

Within 15 to 25 minutes an amount of methemoglobin equal to 20 to 22 per cent of the total hemoglobin was completely removed from

the blood so that none could be found in it spectroscopically. At least part of the methemoglobin was excreted in the urine.

Storage of Methemoglobin.—Extracts of the various organs made immediately post mortem revealed no evidence that methemoglobin is accumulated in any one place. When the methemoglobin is present in the red blood cells only, even when all the hemoglobin is changed to methemoglobin, with death by virtual suffocation due to lack of labile oxygen, the plasma is always free from methemoglobin bands.

The methemoglobin produced in the red blood cells is rapidly destroyed. In the milder cases of experimental methemoglobincythemia this destruction was found to be so rapid that repeated and careful examinations of blood cells, plasma, urine, and tissues failed to reveal its presence.

Table II gives the results from rabbits injected with solutions of potassium ferricyanide. The action of this substance *in vivo* is relatively slow, but by repeated injections over a period of several hours almost 50 per cent of the hemoglobin may be changed. In the three rabbits the potassium ferricyanide changed 43, 30, and 19 per cent respectively of the hemoglobin, doubtless into methemoglobin, and yet the latter was so rapidly destroyed that practically none of it could be found in the blood.

In another rabbit (Table III) within 5 days the hemoglobin diminished 39 per cent, yet no methemoglobin was found spectroscopically in either plasma or cells. Moreover, the urine and the aqueous extracts of liver, lungs, spleen, kidney, heart, skeletal muscle, intestines, and feces showed no methemoglobin by spectroscope.

The rapid production of methemoglobin, as by the injection of sodium nitrite, by which an easily controllable degree of methemoglobin formation may be brought about, gives the same results.

In Rabbit 7 (Table IV) practically all the hemoglobin was changed to methemoglobin, the animal dying immediately of suffocation. The pigment is wholly in the cells. Rabbit 8 lived for 26 minutes with hemoglobin of only 2.9 gm. per 100 cc. of blood, or 23 per cent of the initial value. Yet within this short time the methemoglobin had decreased to 6.7 gm. per 100 cc. of blood. A rabbit of 2.85 kilos has 142 cc. of blood (5 per cent by weight (Van Slyke and Salvesen, 1919)), or in this case 18.1 gm. of hemoglobin, of which at death 4.1

TABLE II.

Production of Methemoglobin in Rabbits by Potassium Ferricyanide.

Rabbit No.	Date.	Time.	Injection.	Hemoglobin per 100 cc. of blood.	Methemoglobin per 100 cc. of blood.
	1919	*a.m.*		*gm.*	*gm.*
3	Apr. 25	9.30		15.2	
		10.15	10 cc. of 0.02 M potassium ferricyanide intravenously.		
		10.30		13.2	
		11.50	10 cc. of 0.02 M potassium ferricyanide intravenously.		
		p.m.			
		12.15		11.0	
		2.45	5 cc. of 0.02 M potassium ferricyanide intravenously.		
		3.15		8.8	
	Apr. 29			8.9	
	May 6			8.7	0.0
		a.m.			
4	May 7	11.35		11.1	
		11.45	5 cc. of 0.02 M potassium ferricyanide intravenously.		
		p.m.			
		12.45		10.5	0.0
		1.02	5 cc. of 0.02 M potassium ferricyanide intravenously.		
		2.15		10.0	0.3
		2.30	5 cc. of 0.02 M potassium ferricyanide intravenously.		
		2.31	(Animal died.)	7.8	0.2
		a.m.			
5	May 9	9.20		13.3	
		9.40	20 cc. of 0.02 M potassium ferricyanide intravenously.		
		9.45		14.1	0.0
		10.30		13.0	0.4
		11.45		12.5	0.0
		p.m.			
		2.07	10 cc. of 0.02 M potassium ferricyanide intravenously.		
		2.30		10.8	0.0

TABLE III.

Production of Methemoglobin in Rabbits by Potassium Ferricyanide.

Rabbit No.	Date.	Time.	Injection.	Hemoglobin per 100 cc. of blood.	Methemoglobin per 100 cc. of blood.	Methemoglobin spectrum.	
						Plasma.	Cells.
	1919	*m.*		*gm.*	*gm.*		
6	June 5	12.00 *p.m.*					
		2.27	10 cc. of 0.02 M potassium ferricyanide intravenously.	11.6*			
		a.m.					
	June 6	10.00	5 " " 0.02 M " " "				
		11.07	5 " " 0.02 M " " "				
		11.47	5 " " 0.02 M " " "				
		p.m.					
		12.21	5 " " 0.02 M " " "				
		1.45	5 " " 0.02 M " " "				
		2.47	5 " " 0.02 M " " "				
		4.00	5 " " 0.02 M " " "				
		a.m.					
	June 7	10.58	5 " " 0.02 M " " "				
		11.45	5 " " 0.02 M " " "				
		p.m.					
		1.15	5 " " 0.02 M " " "	8.2	0.2		
	June 9	3.40	10 " " 0.02 M " " "				
		4.32	10 " " 0.02 M " " "				
	June 11	4.30	(Killed.)†	7.0	0.5	Negative.	Negative.

* Colorimetric determination.

† Extract of liver, lungs, spleen, heart, skeletal muscle, intestines, and feces showed no methemoglobin spectrum. Urine negative also.

gm. were left. Of the 14 gm. of methemoglobin formed, only 9.5 gm. remained at death. In other words, 4.4 gm. of methemoglobin were destroyed in 26 minutes. In Rabbit 9, 1.8 gm. of methemoglobin were destroyed in 30 minutes. In all these rabbits no methemoglobin was found in the plasma, although the blood was dark chocolate in color; it was present in the cells only.

Therefore even in extreme cases of methemoglobin production in which death results quickly from an insufficient oxygen supply, there is a rapid destruction of methemoglobin. When the methemoglobin is produced more slowly this destruction is fast enough to prevent its accumulating in the blood in sufficient quantity to be detected by the spectroscope. Not only is this true when chemicals are the causative agent, but also when methemoglobin-producing bacteria act *in vivo*.

However, when the production of methemoglobin is very extensive, *e. g.* 30 to 50 per cent of total pigment, and sudden, as following intravenous injections of sodium nitrite, methemoglobin may be found in the blood, but always in the red blood cells. Even in these instances, if the animal survives for a comparatively brief time, the methemoglobin disappears from the blood.

The mechanism of this disappearance has not been determined. Pearce, Austin, and Eisenbrey (1912) studied the fate of hemoglobin injected intravenously and found that no hemoglobin was eliminated through the kidney unless the rate of injection was above a minimum which was high, but that nevertheless the injected hemoglobin rapidly disappeared from the blood stream. Furthermore, Whipple and Hooper (1913) showed that injected hemoglobin is changed rapidly to bile pigments in normal dogs and in dogs with Eck fistula or hepatic ligation. Since methemoglobin is closely related chemically to hemoglobin it is possible that it is similarly disposed of.

Action of Pneumococci on Hemoglobin in Vivo.—Grüter (1909) inoculated a cat intraperitoneally with a large amount of pneumococcus culture but was unable to demonstrate methemoglobin in the blood. Peabody (1913) developed the subject further by inoculating rabbits intravenously with the organisms from 300 to 600 cc. of 24 hour broth cultures of pneumococci. Death resulted within a few hours and the blood always showed on direct film enormous num-

TABLE IV.

Intravenous Injection of Sodium Nitrite in Rabbits. Rapid Formation and Disappearance of Methemoglobin.

Rabbit. No.	Weight.	Time.	Injection.	Hemoglobin per 100 cc. of blood.	Methemoglobin per 100 cc. of blood.	Methemoglobin* destroyed.	Methemoglobin spectrum.		
							Plasma.	Cells.	Urine.
	kg.	*a.m.*		*gm.*	*gm.*	*gm.*			
7		9.15		7.8					
		9.30	0.95 gm. of sodium nitrite intravenously.						
		9.31	(Died.)	0.3			Negative.	++++	Negative.
		p.m.							
8	2.85	3.00		12.7	0.0				
		3.21	0.1 gm. of sodium nitrite per kilo intravenously.						
		3.47	(Died.)	2.9	6.7	4.4	Negative.	++++	Negative.
		a.m.							
9	1.7	9.00		10.0	0.0				
		9.30	0.12 gm. of sodium nitrite per kilo intravenously.						
		10.00	(Died.)	3.6	4.3	1.8	Negative.	++++	Negative.

* Estimated on the assumption that the blood volume is 5 cc. per 100 gm. of body weight (Van Slyke and Salvesen, 1919).

bers of pneumococci. With these overwhelming pneumococcemias Peabody showed that there was a rapid fall in total hemoglobin as measured by oxygen capacity, but he was rarely able to demonstrate the presence of methemoglobin in the blood. He nevertheless concluded that this fall in total oxygen capacity (hemoglobin) was due to a methemoglobin production. However, when these experiments are repeated with quantitative determination of methemoglobin, it is clear that bacterial methemoglobinemia is similar to that produced chemically in that the methemoglobin formed rapidly disappears.

Rabbits were inoculated by ear vein with the centrifuged pneumococci from 24 hour cultures resuspended in a small amount of saline solution. Death occurred in all cases and all showed by direct film of the blood enormous numbers of organisms. The results are shown in Table V.

In Rabbit 10, after 5 hours, despite a fall in hemoglobin of 3.94 gm. per 100 cc. of blood (30 per cent of the total), there was no methemoglobin in the blood. In No. 12, 3.82 gm. of hemoglobin per 100 cc. of blood (30 per cent of the total) were changed, but only 0.83 gm. of methemoglobin per 100 cc. was found. In Nos. 10 and 11 at death considerable amounts of methemoglobin were found in the blood, but amounts representing only 30 to 32 per cent of the total change. It is clear then that the mechanism here is the same that is described in the production of methemoglobin by chemicals. In the gradual change of hemoglobin to methemoglobin the latter pigment disappears as fast as it is formed, so that it is never present in the blood in sufficient concentration to be detected spectroscopically. Only near death does the rapid production of methemoglobin by the huge numbers of pneumococci lead to its accumulation in the blood in quantities greater than the now disordered metabolism can handle. In other words, it is rarely possible to find methemoglobin in the blood, unless just before death, even after considerable changes of hemoglobin to methemoglobin.

It is to be further noted that the methemoglobin present in these instances is entirely intracellular. Examination of the plasma spectroscopically gives negative results, but the centrifuged cells in the last samples of Nos. 11 and 12 contain abundant methemoglobin. The urine also is always negative. The condition is a methemoglobincythemia.

TABLE V.

Production of Methemoglobin by Pneumococci in Rabbits.

Rabbit No.	Date.	Time.	Conditions.	Hemoglobin per 100 cc. of blood.	Hemoglobin decrease per 100 cc. of blood.	Methemoglobin per 100 cc. of blood.	Remarks.
	1919	*a.m.*		*gm.*	*gm.*	*gm.*	
10	June 15	11.45		12.98	0.0	0.0	
		p.m.					
		1.00	Inoculated.				
		3.30		10.82	2.16	0.30	Blood bright red.
		5.00		9.04	3.94	0.00	" " "
		8.45	Very sick.	6.02	6.96	2.09	" chocolate-colored.
		9.00	Died.				
		a.m.					
11	June 25	9.00		12.78		0.0	
		10.25	Inoculated.				
	June 26	10.25		11.60	1.18	0.18	Blood bright red.
		p.m.					
		4.19	Died.	5.75	7.03	2.27	" chocolate-colored.
		a.m.					
12	June 30	9.00		12.87		0.0	
		10.50	Inoculated.				
		p.m.					
		3.30	Died.	9.05	3.82	0.83	Blood bright red.

Rabbit 10 received Pneumococci Type I from 250 cc. of broth cultures. Rabbit 11 received Pneumococci Type I from 100 cc. of broth cultures. Rabbit 12 received Pneumococci Type II from 150 cc. of broth cultures. Postmortem films of blood from all the rabbits showed enormous numbers of pneumococci.

Non-Production of Methemoglobin by Pneumococcus Autolysates.

Grüter (1909) observed methemoglobin formation in the presence of dead cultures, filtrates, and centrifugates. Butterfield and Peabody (1913) also have produced methemoglobin by the autolysates of pneumococcus cultures. Rieke (1904), however, attributed methemoglobin formation to the living pneumococcus and streptococcus only. Cole (1914) likewise reached the same conclusion, since he was unable to demonstrate the production of methemoglobin by filtrates and extracts of pneumococcus cultures.

We have also been unable to show a transformation of hemoglobin to methemoglobin without the living organism. A broth culture of pneumococcus was used in which, after 5 days incubation, all organisms were autolyzed as shown by sterile subcultures. Prepared in this way the pneumococcus autolysate failed to produce methemoglobin when mixed with hemoglobin solutions, as shown in Table VI.

It is quite possible, however, that if, as has been suggested by Avery and Cullen,[1] the proper conditions are found for the reaction or if concentrated solutions of intracellular products obtained by the disintegration of the pneumococcus are used, methemoglobin formation without the living organism may be demonstrated.

TABLE VI.

Action of Autolysate of Pneumococci on Hemoglobin.

Tube No.	Conditions.	Broth.	Autolysate.	Hemoglobin solution.	Initial hemoglobin.	Hemoglobin after 20 hrs.	Methemoglobin after 20 hrs.
		cc.	*cc.*	*cc.*	*per cent*	*per cent*	*per cent*
1	Room temperature.....	0	10	10	7.14	6.45	0.69
2	Ice box.....	0	10	10	7.14	7.14	0.00
3	Room temperature.....	10		10	7.30	6.67	0.63

DISCUSSION.

Methemoglobin Formation in Pneumonia.—Peabody (1913) was the first to explain fall of blood oxygen capacity observed in some (usually fatal) pneumonia cases as due to a change of hemoglobin to methemoglobin. He suggested that this alteration could take place through the action of a soluble product of bacterial metabolism. If the production of methemoglobin by soluble bacterial products, as suggested by Peabody, is substantiated by further experiments, then it is possible that the decrease in hemoglobin may be brought about by the escape of these products into the blood stream from a focus of pneumococcic infection in the lungs. However, all the cases studied by Peabody (1913) and Harrop (1919) with a sudden and well defined decrease of hemoglobin had a pneumococcemia of profound degree (from 2,500 to 16,000 colonies per cc. of blood). It is, therefore,

[1] Avery, O. T., and Cullen, G. E., unpublished work.

probable that a great production of methemoglobin occurs only when the opportunity for the direct action of the pneumococci on the blood is greatest; *i. e.*, in cases with a bacteremia.

Methemoglobin and Cyanosis in Pneumonia.—Although there has been a prevalent belief that methemoglobinemia is a factor in the cyanosis of pneumonia, many observers (Abrahams, Hallows, and French, 1919; Synnott and Clark, 1918; and Peabody, 1913) have looked for it spectroscopically in cases of pneumonia deeply cyanotic, but have failed to find it.

The absence of methemoglobinemia in pneumonia, even when there is a marked fall in oxygen capacity, is, we believe, explainable by the results reported in this paper. Methemoglobin disappears from the circulation with great rapidity, whether it is introduced by injection of methemoglobin or formed within the circulation by the action of chemicals or of pneumococci. If we take into consideration this fact, the ability of the pneumococcus to form methemoglobin, and the consistent failure to find it in the blood in the cyanosis of pneumonia, it appears that the following conclusions represent the most probable explanation of what occurs.

CONCLUSIONS.

In the occasional cases of pneumonia which show a decrease in the oxygen capacity of the blood, the decrease is probably due to a formation of methemoglobin. The latter is removed from the circulation, however, as rapidly as it is formed, so that it can seldom be detected even qualitatively, and is probably never a cause of cyanosis.

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FIBRIN AND SERUM AS A CULTURE MEDIUM.

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INTRODUCTION.

Various artificial media have been used so far in the cultivation of tissues. Lewis and Lewis used bouillon and agar,[1] salt solutions,[2] and other media of known chemical constitution,[3] but the growth was by no means equal to that in plasma or lymph, either in extent or duration. Swezy[4] obtained cell proliferation of chick embryo heart tissue in a medium composed of egg albumin and muscle extract. Carrel and Burrows[5] found homogenic as well as heterogenic serum a useful culture medium for embryonic chick tissue. Ingebrigtsen[6] studied the growth of tissue outside the organism in a medium composed of agar and serum. Smyth[7] devised a so called simplified medium composed of agar and trypsinized peptone solution for embryonic tissue culture. No attempt was made to undertake a comparative quantitative study of the amount of tissue produced in these different media. Burrows[8] pointed out that embryonic tissues grew as well in saline solution as in plasma. He thought that growth consisted in a spreading of the cells and not in any increase in the mass of tissue, and that it appeared to take place at the expense of the original fragment. It is certain that the tissues did not increase

[1] Lewis, M. R., and Lewis, W. H., *Bull. Johns Hopkins Hosp.*, 1911, xxii, 126.

[2] Lewis, M. R., *Anat. Rec.*, 1915–16, x, 287. Lewis, M. R., and Lewis, W. H., *J. Am. Med. Assn.*, 1911, lvi, 1795.

[3] Lewis, W. H., and Lewis, M. R., *Anat. Rec.*, 1912, vi, 207.

[4] Swezy, O., *Biol. Bull.*, 1915, xxviii, 47.

[5] Carrel, A., and Burrows, M. T., *J. Exp. Med.*, 1911, xiv, 244.

[6] Ingebrigtsen, R., *J. Exp. Med.*, 1912, xv, 397–398.

[7] Smyth, H. F., *J. Med. Research*, 1914–15, xxxi, 255.

[8] Burrows, M. T., *Tr. Cong. Am. Phys. and Surg.*, 1913, ix, 77.

in mass in any of the artificial media, nor could they be kept alive after a certain time. Therefore, these media could not be used for a quantitative study of the problem of growth.

It is known that the presence of embryo juice in adult plasma allows an indefinite growth of the fibroblasts and an increase in the mass of tissue. But the composition of this medium is complex and cannot be modified easily. It would be useful to find a medium endowed with the same properties as plasma and embryo juice, and more adaptable to the nature of the experiment.

The purpose of this article is to describe a technique for preparing a medium composed of fibrinogen, serum, and tissue juice, and to compare the growth of fibroblasts in this medium with that obtained in a medium composed of plasma and embryo juice.

EXPERIMENTAL.

The technique of Mellanby[9] was used in the preparation of fibrinogen. 10 cc. of normal adult chicken plasma were diluted with 90 cc. of sterile distilled water, and thoroughly shaken in an Erlenmeyer flask; 1 cc. of a 1 per cent acetic acid solution was added, drop by drop, and at the same time the mixture was agitated. The precipitate was allowed to settle partially in the cold for about 1 hour. The contents of the flask were then shaken and poured into centrifuge tubes, 25 cc. in each tube. After 10 minutes centrifugation, the supernatant fluid was decanted. The tubes were inverted over a sterile piece of filter paper for complete drainage. The precipitate contained in each centrifuge tube was combined and made up to 2.5 cc. with sterile, distilled water. When thoroughly mixed, it had the appearance of rich milk; on standing, a heavy sediment settled, superimposed by a layer of turbid fluid. Equal volumes of this suspension and Ringer's solution formed a slightly hazy, firm homogeneous clot after about 4 minutes. With an equal volume of serum, the mixture formed a clear fluid which did not coagulate after 10 minutes, but on the addition of a trace of embryonic tissue juice, coagulation occurred rapidly. The hydrogen ion concentration of such a preparation of fibrinogen was between 6 and 6.3. A mixture

[9] Mellanby, J., *J. Physiol.*, 1917, li, 396.

of 12.5 per cent fibrinogen suspension, 37.5 per cent chicken serum, and 50 per cent embryonic tissue juice, had a pH of between 7 and 7.3. Such a preparation coagulated in about 1 minute.

The experiments were begun with 48 hour cultures, derived from a strain of connective tissue in its 9th year *in vitro*.[10] Each fragment of tissue was divided in two parts and washed in Ringer's solution for about 40 seconds. One fragment was then cultivated in equal volumes of normal chicken plasma and embryonic tissue juice. The other fragment was cultivated in the experimental medium composed of one-fourth volume of fibrinogen suspension, three-fourths volume of chicken serum, and one volume of embryonic tissue juice. The constituents of this medium were first mixed by drawing them up

TABLE I.

Experiment No.	Culture No.	First passage.			Second passage.			Third passage.			Fourth passage.		
		Relative increase.		Ratio, $\frac{E}{C}$.	Relative increase.		Ratio, $\frac{E}{C}$.	Relative increase.		Ratio, $\frac{E}{C}$.	Relative increase.		Ratio, $\frac{E}{C}$.
		Control.	Experiment.		Control.	Experiment.		Control.	Experiment.		Control.	Experiment.	
1	17555	17.5	16.2	0.93	12.5	11.6	0.93	10.3	9.2	0.89	11.0	8.9	0.81
											9.7	8.0	0.83
2	17536	21.5	18.3	0.85	21.7	19.3	0.88	10.5	8.0	0.76	10.0	9.2	0.92
					21.0	14.6	0.70	10.7	8.8	0.82	8.4	Liquefied.	

and expelling them from a bulb pipette, after they had been dropped into the hollow of a deep, concave slide. After 48 hours the tissues were washed and cultivated in the same medium.

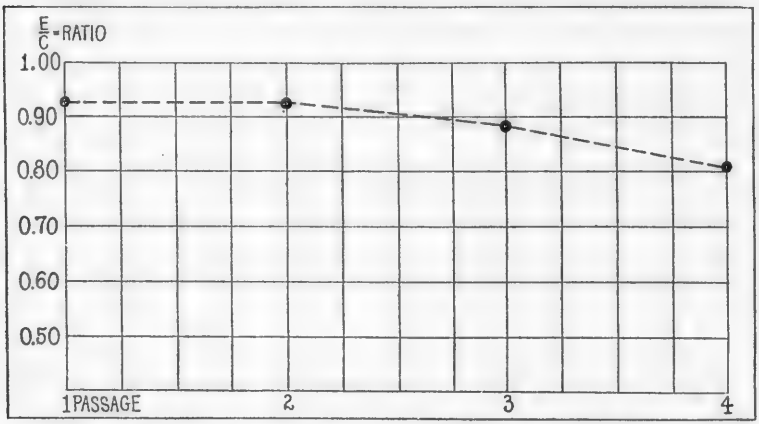
The cultures were incubated at 39°C. Observations were made on the coagulation time, consistency, duration of the coagulum, and character of growth as compared with the controls. The cultures were traced, measured, and the relative increase was calculated, according to the technique mentioned in a previous article.[11]

The technique was developed in the course of thirty-five experiments. The results do not require detailed description because the

[10] Ebeling, A. H., *J. Exp. Med.*, 1919, xxx, 531.

[11] Ebeling, A. H., *J. Exp. Med.*, 1919, xxx, 533–534.

appearance of the coagulum and of the growth of the strain of fibroblasts was about the same as that of the control in plasma and embryo juice. Generally, the width of the zone of new tissue and its density were slightly less in the experiment than in the control; the difference in most instances was approximately 10 per cent. In other experiments the tissues were allowed to grow for 48 hours several times, and then transplanted into a medium of the same composition. The results of two of these experiments are given in Table I and in Text-figs. 1 and 2. In the table the figures for the relative growth of the tissue obtained in the control and experiment represent the amount

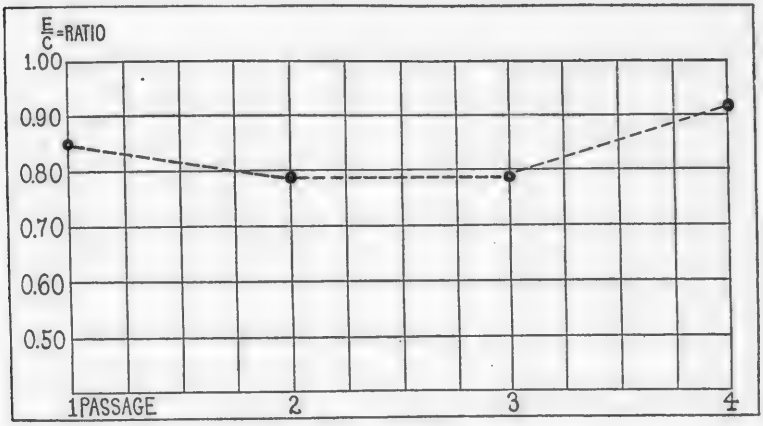


TEXT-FIG. 1. Experiment 1. Ratio between the two relative growths, experiment and control.

of growth which was obtained during the time interval chosen (48 hours), expressed in function of the area of the primitive fragment of tissue. The ratio between the two relative growths, $\frac{\text{Experiment}}{\text{Control}} = \frac{E}{C}$, is also given. In Experiment 1 the tissues of both experiment and control were divided after the third passage. In Experiment 2 the tissues were divided in the same way after the first passage.

There is a close relation between the relative growths of the experiments and of the controls. This relation, expressed as ratios in the first experiment, was 0.93, 0.93, 0.89, 0.81, 0.83; and in the second,

0.85, 0.88, 0.70, 0.76, 0.82, 0.92. These figures express the fact that in the time elapsed between two passages, the amount of growth is very nearly the same both in the experiment and the control. A curve was plotted for both experiments in which these ratios were expressed in ordinates and the number of passages (48 hour intervals) in abscissæ.



TEXT-FIG. 2. Experiment 2. Ratio between the two relative growths, experiment and control.

DISCUSSION.

A heavy precipitate could be obtained from 10 cc. of diluted plasma by adding 1.2 to 1.6 cc. of a 1 per cent acetic acid solution. But the final product was too acid. 1 cc. of the acetic acid solution produced a precipitate which, after suspension in distilled water, had a hydrogen ion concentration between 6 and 6.3. The hydrogen ion concentration of serum was from 8 to 8.3 and that of embryo juice from 7.3 to 7.5. When the precipitate suspension was dissolved in serum and mixed with embryo juice, the hydrogen ion concentration of the mixture varied from 7.3 to 7.5.

The coagulation was brought about by the addition of embryo juice. The addition of calcium was not essential to promote coagulation. On the contrary, a precipitate formed and liquefaction of

the coagulum occurred after 24 hours. The presence of serum is generally necessary to prevent liquefaction of the coagulum. In the experiments in which fibrinogen suspension was mixed with Ringer's solution or embryo juice alone, without serum, coagulation took place. But progressive liquefaction began soon afterwards. When serum was added in small quantities to fibrinogen suspension and embryo juice, the stability of the clot could be maintained.

The suspension of fibrinogen was used in various concentrations. If the medium contained more fibrinogen than plasma, the tissues did not grow well. When the concentration of fibrinogen was decreased, the coagulum was not dense enough and liquefaction often occurred within 24 hours.

It was found that excellent growth took place in a medium composed of 12.5 per cent fibrinogen suspension, 37.5 per cent serum, and 50 per cent embryo juice. Coagulation occurred within 1 minute and the coagulum was still firm after 48 hours.

The character of the growth of the old strain of connective tissue in the medium was not different from that observed in plasma. The growth was slightly less extensive in the experimental medium than in the control. It was easy to extirpate the fragment of tissue from its medium after 48 hours, and to transplant it into another medium. After every passage the amount of new tissue was about as large in the experiment as in the control. There was no doubt that the culture could have been kept alive for several more generations. This shows the possibility of keeping a strain of connective tissue in a medium composed of serum, fibrinogen, and embryo juice in about the same condition as in plasma and tissue juice.

CONCLUSIONS.

A technique is described by which a medium composed of fibrinogen suspension, serum, and embryo juice may be made.

Fibroblasts grew in this medium about as well as in plasma and embryo juice.

A strain of connective tissue in this medium remained practically as active as the control for several passages.

FLAGELLUM OF THE MICROORGANISM OF RAT-BITE FEVER.

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PLATES 81 AND 82.

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Since 1915 when Futaki and Ishiwara found, independently, a microorganism as the cause of rat-bite fever, various investigators have published their results on this subject. The present paper is a report of observations recently made by the writer on the microorganism.

Material.

Two strains were kept in the laboratory, one from an infected child, and the other from a rat. Besides these, four were obtained from fourteen rats. Each of these strains was injected cutaneously into two guinea pigs. In a few days the site of injection became hard, the lymphatic glands were swollen, and the animals developed fever and died. Death was caused by the injected organism.

Method.

The blood of mice and guinea pigs injected with the strains described above was observed under dark-field illumination. The flagellum of the microorganism was fixed best with vapor of osmic acid (Figs. 1 to 4). Fixation with bichloride of mercury was unsuitable, since it caused contraction. Fixation with methyl alcohol was simple and brought out clearly a large flagellum. Before the cover-glass preparation was dried it was exposed to the vapor of osmic acid (1 gm. of osmic acid + 100 cc. of distilled water + 10 drops of 5 per cent bichloride of mercury) for from 30 seconds to 1 minute. Giemsa's solution or a modified reagent was used for staining. Gen-

erally the microorganism took a long time to stain, though the time required depended upon the power of the solution, which varied with the proportion of its components, "methylene azur," methylene blue, and eosin. The proportion of the components of the solution was not quite the same as that of Giemsa's solution, which did not give the best results, the commercial product often failing to stain the flagellum. A better solution for staining was obtained by adding a little alkali to Akashi's solution which is a modification of Giemsa's stain. It is worth mentioning that not only did the staining power of the preparation become very poor a few weeks after it was made, but also it was not always possible to obtain the same staining power, though the method of preparation was always the same. This point will be studied further.

OBSERVATIONS.

The microorganism consisted of the body and the flagella, as first recognized by Ishiwara and his coworkers and confirmed by many investigators. The body was short and thick; there were two or three windings which were thick, regular, and spiral, but not wave-like. Its length was about 3 microns. The microorganism moved energetically among the blood corpuscles when it was observed under the dark-field microscope, but its form was not changed during movement.

As to the number of the flagella, most writers have observed one flagellum at one end or at both ends. Matsusaki and his coworkers and Otawara observed one at one end and two at the other. In stained preparations the flagellum was observed at both ends of the microorganism or sometimes at one end only. In fresh preparations under dark-field illumination, however, they could always be seen at both ends. It is probable, therefore, that the flagella are generally present at both ends. In preparations fixed with methyl alcohol I found one flagellum at one or both ends. But in one preparation fixed with vapor of osmic acid, one flagellum was shown at one or both ends of some microorganisms, while many flagella were detected at one or both ends of others. These two types of microorganisms were always found by this method of staining, and the better the staining power of the solution the greater was the number with many

flagella. This was true even in the same material and preparation and seems to indicate that the number of flagella demonstrable depends on the staining power of the solution. These facts were confirmed by a study of the six strains already mentioned. The largest number of flagella counted was seven at one end. There were a few instances in which more than seven flagella were present, but they were so dense and tangled that they could not be counted. On the other hand, in one type of microorganism which showed one stained flagellum at one or both ends of the body, only one flagellum was found even when the staining power of the solution was best.

It therefore seemed advisable to investigate further the relation of these two types. 100 microorganisms with one flagellum at one or both ends and 100 with two or more flagella at one or both ends were studied. It was found that there was no relation between the length of the body or number of windings and number of flagella. It seemed possible, however, that the number of flagella might be related to stages of development. With this idea in mind streak preparations of blood taken from the ear vein of infected guinea pigs were made. Even in these cases two types were always found; that is, some microorganisms had one large flagellum at one or both ends and others had two or more flagella at one or both ends. But the ratio of the two types of microorganisms during one period showed no special relation to that at any other period.

The diameter of the end of the body of the microorganisms with one large flagellum gradually decreased to the flagellum, while in specimens with two or more slender flagella the ends of the body were blunt. Sometimes it was observed, however, that those with one slender flagellum were also blunt at the end of the body. In this case the number of flagella which were attached at the blunt end increased with better staining.

There were some peculiar specimens in which two flagella were fused into one (Fig. 8, *a* and *b*). In some instances the flagellum was divided into two or three parts at certain points (Fig. 8, *d* to *f*). Or again one flagellum was divided into two or three branches and one of the branches was again divided into two (Fig. 8, *f*). The part of the flagellum which was divided into two or three was generally

thicker than any other part. Furthermore, the diameters of the flagella of a microorganism might vary. In this case one flagellum which was larger than the others was sometimes divided into two or three (Figs. 5, *e*, 8, *d* and *f*, and 10, *a*).

Generally the flagellum was not straight; in the living specimen it was spiral. If the object-glass was skillfully moved in following a swift movement of the microorganism so as to keep it in the center of the dark-field, the movement became slow and the winding of the flagellum was readily observed, as the microorganism was weak to resist direct light; motion ceased even though the light was removed. In this case the flagellum became comparatively straight, L-shaped, or bow-like instead of spiral. Such was always true of stained preparations fixed with methyl alcohol. After fixation with osmic acid the flagellum was generally spiral just as in living microorganisms. Often the direction of the spiral was the same as that of the winding of the body. The spiral consisted of one and a half or two windings. When many flagella occurred at one end, the spiral of each was independent of the others. The direction of the spirals differed, but in many instances they faced one another. Two flagella, for example, which were extended at one end, were situated at an angle of 180° to each other (Fig. 5, *b* and *c*), or three flagella were kept at 120° (Figs. 5, *d* and 8, *e*).

The direction of the flagellum from the body varied with the movement. Futaki observed in streak preparations that the direction of the flagellum varied and thought that it was due to technique. This is not, however, always the case, because variation is observed even in a fresh preparation seen under the dark-field microscope. Often, however, the flagellum was extended in the same direction as the body as observed in a forward or backward movement (Figs. 1 and 5, *a*), though this did not always occur (Fig. 6, *a* to *c*). In some instances the flagellum was tangled around the body (Fig. 7, *b* and *c*), so that the microorganism appeared to have an undulating membrane. Furthermore, sometimes it was noticed that long microorganisms had one or two flagella in the center or near the center of the body. The center from which the flagellum originated was generally, though not always, small in comparison with other parts of the body. Moreover, this part was commonly slightly bent. In addition, two micro-

organisms, each of which had two or three windings, were connected with a thread and a flagellum at the connected ends (Fig. 10, *c* and *d*). Short microorganisms with one or more flagella at one or both ends were often observed (Fig. 9, *c* and *d*). These facts suggest that the microorganism divides transversely, though it is generally assumed that it divides longitudinally.

CONCLUSIONS.

The work of previous investigators was confirmed in that the microorganism of rat-bite fever was found to have flagella which are clearly visible by dark-field illumination and which can be stained. The best staining is obtained with alkalinized Akashi solution, which is a modification of Giemsa's solution; the vapor of osmic acid gives the best fixation.

The number of demonstrable flagella seems to vary with the technique. Sometimes many slender flagella unite into one large one or one large flagellum divides into several smaller ones. It may be concluded that commonly many slender flagella occur at the ends and that these may unite into one or several large flagella. In the living microorganism the flagella appear to be spiral. Their form in fixed preparations depends upon the method employed. Some long forms have flagella arising at the middle of the body; this seems to indicate that division is transverse and not longitudinal as generally believed. The rigid body, the signs of transverse division, and multiple flagella seem to distinguish the forms reported here from spirochetes and indicate that they are spirilla.

The work was done under the direction of Professors Miyairi and Ogawa.

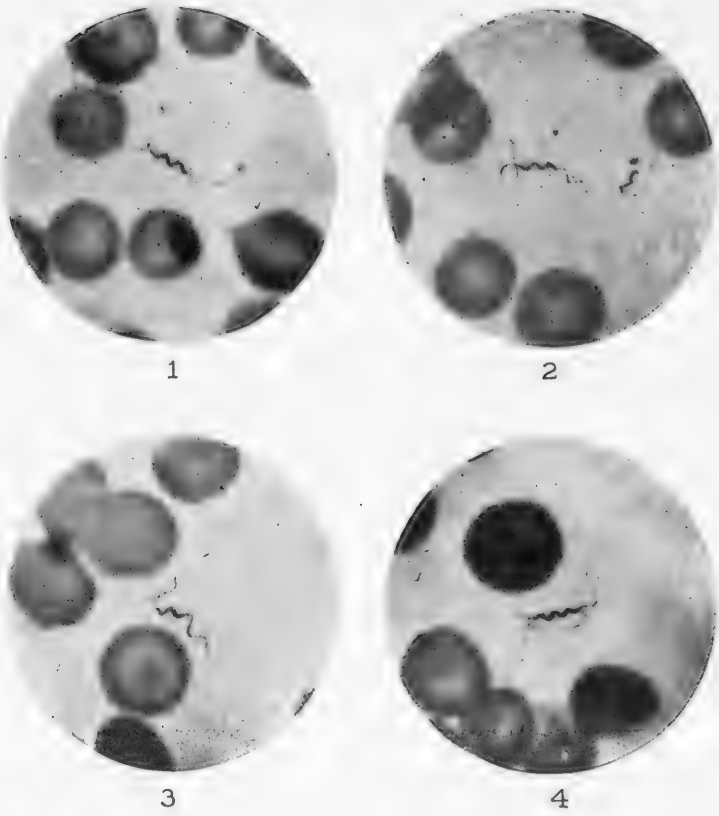
EXPLANATION OF PLATES

PLATE 81.

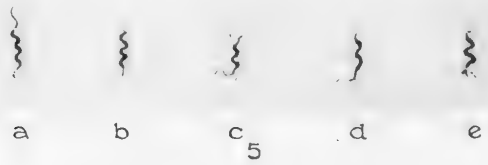
FIGS. 1 to 4. Photographs of microorganisms which were found in the blood of mice previously injected. Vapor of osmic acid fixation. × 2,000.

PLATE 82.

FIGS. 5 to 10. Free-hand drawings of microorganisms found in the blood of mice and guinea pigs previously injected. All flagella are drawn on the same plane, though they were not so in reality.



(Adachi: Microorganism of rat-bite fever.)



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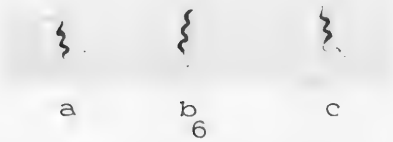

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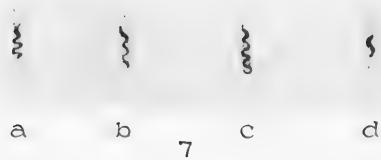

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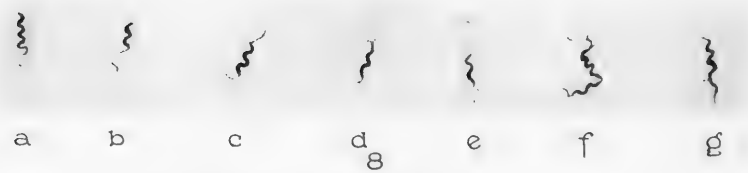
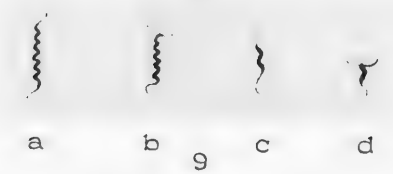


8



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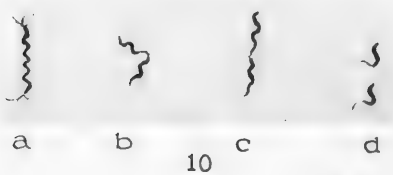

10

(Adachi: Microorganism of rat-bite fever.)

IMMUNOLOGIC STUDY OF STRAINS OF BACILLUS PFEIFFERI ISOLATED FROM A CASE OF MENINGITIS.

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The discovery of *Bacillus pfeifferi* by Pfeiffer in 1892, although made after the pandemic of 1899–90 had spent its force, was followed by rather general acceptance of the etiological relation of the organism to the disease. In the two succeeding decades it was found to have a widespread distribution in the upper air passages of normal individuals. In the next decade there were a number of minor and mild epidemics, in several larger American cities, of a disease clinically like influenza in which, not the influenza bacillus, but the streptococcus appeared to be the causal agent. These epidemics, however, were not associated with the dangerous complications nor did they have the high mortality of true epidemic influenza. With the passage of time since the 1899–90 pandemic the position of *Bacillus pfeifferi* as a pathogenic microorganism had become one of minor importance when the last pandemic made its appearance in 1918. The large amount of bacteriologic work done throughout the world in this epidemic still leaves the part played by *Bacillus pfeifferi* as the causative agent of pandemic influenza in doubt. Immunologic investigations have not helped to clear up this doubt.

That *Bacillus pfeifferi* may have well marked invasive and pathogenic properties is established by its occurrence as the only microorganism in a small proportion of cases of secondary bronchopneumonia and especially by its relation to sporadic cases of acute purulent meningitis.

Kuskow,[1] in 1895, estimated suppurative meningitis in the 1899–90 epidemic at 2.5 to 5 per cent of the fatal cases. That the reported cases from which he de-

[1] Kuskow, N., *Virchows Arch. path. Anat.*, 1895, cxxxix, 406.

rived these figures were due to *B. pfeifferi* was not established by bacteriologic examination, and his figures cannot be accepted as giving the true incidence of meningitis in influenza. In 1904 Jundell[2] reported two cases and found in the literature twelve cases from which pure cultures of the bacillus were obtained. These cases dated from 1895. In those previous to this date the organism was not cultivated or the cultures were impure. In 1911 Wollstein[3] reported 49 cases, eight being cases studied by herself. There was a further group of nine reported cases in which the cultures were contaminated, and a group of eight which she considered doubtful. Torrey[4] reported two cases in 1916, making a total of 82 certain cases recorded up to that time; of these cases eight recovered. Lacy[5] in 1918 reported one case in which the organism was cultivated from the meninges at autopsy but not from the spinal fluid during life, and another case in which *B. pfeifferi* was grown in pure culture from the spinal fluid. Additional instances are reported by Nyberg,[6] Brown,[7] and Moody,[8] each of whom reported two cases; and by Aaser,[9] Packard,[10] Bhat,[11] and Hills,[12] each of whom reported one case.

The important point to be noted in connection with these 94 cases is that they occurred during the period between the two pandemics of 1899–90 and 1918–19. The small number of cases reported since the last epidemic began and experience in civil and military hospitals during the epidemic indicate that meningitis due to *Bacillus pfeifferi* is a sporadic infection of not very great frequency. While there seems to have been some increase in meningitis due to *Bacillus pfeifferi* since the height of the epidemic, in view of the widespread distribution of this organism the increase would appear not so great as might be expected if *Bacillus pfeifferi* were the cause of epidemic clinical influenza. Perhaps this fact may have some bearing upon any attempt to determine the part played by *Bacillus pfeifferi* in epidemic clinical influenza. Other points of interest which appear from the

[2] Jundell, I., *Jahrb. Kinderheilk.*, 1904, lix, 777.
[3] Wollstein, M., *Am. J. Dis. Child.*, 1911, i, 42.
[4] Torrey, R. G., *Am. J. Med. Sc.*, 1916, clii, 403.
[5] Lacy, G. R., *J. Lab. and Clin. Med.*, 1918–19, iv, 55.
[6] Nyberg, C., *Finska läk.-sällsk. handl.*, 1915, lvii, 1369.
[7] Brown, A., *Canad. Med. Assn. J.*, 1915, v, 1076.
[8] Moody, E. E., *J. Missouri Med. Assn.*, 1916, xiii, 328.
[9] Aaser, E., *Tidsskr. norske Lægefor.*, 1916, xxxvi, 393.
[10] Packard, F. R., *Ann. Otol., Rhinol. and Laryngol.*, 1916, xxv, 706.
[11] Bhat, K. S., *Lancet*, 1917, ii, 384.
[12] Hills, R., *New York Med. J.*, 1918, cvii, 345.

reported cases are the low age incidence, most of the cases occurring in very young infants or in children and only four of 94 in adults, and the high mortality, only nine of 94 cases having recovered.

OBSERVATIONS.

The isolation of five strains of *Bacillus pfeifferi* from a case of meningitis admitted to the Sarah Morris Hospital for Children gave an opportunity for studying the immunologic reactions of the microorganism as obtained from a single case and for comparing definitely invasive strains with others, perhaps saprophytic, from the upper air passages. The results, together with the recently reported immunologic studies of others, appear to be of fundamental importance for an understanding of the biology of the organism and may have some bearing upon the possibility of developing a therapeutic antiserum.

Isolation of Strains.

The patient, a white, male infant age 17 months, on admission showed vomiting, twitching all over the body, fever, rigidity of the body, discharging right ear, and strabismus. The illness had begun with fever and vomiting 4 days before admission; muscular twitching appeared 2 days and rigidity 1 day before admission; strabismus developed on the day of admission. Death occurred on the 7th day in the hospital. The first culture of *Bacillus pfeifferi* was obtained from spinal fluid removed on the day of admission. This fluid had a cell count of 3,500 and all the characteristics of a fluid from acute purulent meningitis. On the following day cultures were made from the nose, throat, nasopharynx, ear, and blood.

A direct film of the spinal fluid stained by Gram's method showed a Gram-negative pleomorphic organism. There was variation in both size and morphology from coccus-like forms to rather long, slender bacilli. Direct films were not received in the laboratory from the nose, throat, nasopharynx, or ear.

Dewdrop colonies with the typical appearance of *Bacillus pfeifferi* appeared on blood agar cultures from the nose, throat, nasopharynx, blood, and spinal fluid, but not from the ear. Films made from

these colonies appeared, in the case of the spinal fluid culture, as Gram-negative pleomorphic organisms resembling those seen in the direct film. Films made from colonies of the other cultures showed typical, very small, Gram-negative bacilli fairly uniform in size. Subcultures were made on plain agar, Avery's[13] dextrose blood broth, and brown agar. No growth was obtained from the plain agar subcultures. In the Avery broth there was a rather slight growth showing some pleomorphism. In films from the spinal fluid colonies on the brown agar plates, the morphology was more typical than on whole blood agar. Colonies from the other sources tended to become more pleomorphic on brown agar than on whole blood agar. The brown agar was made by adding 2 per cent of defibrinated human blood to plain agar and heating to 80°C. long enough to produce a brown color. The growth on this medium was luxuriant, becoming more so after cultivation, which was in decided contrast to the very fine growth on blood agar. The ear cultures gave only a pure growth of *Staphylococcus albus*. From spinal fluid removed on the day after admission, when the cell count had increased to 10,000, *Bacillus pfeifferi* was again obtained in pure culture. Subcultures from selected colonies of the first spinal fluid culture and of the nose, throat, nasopharyngeal, and blood cultures on brown blood agar and on unheated whole blood agar were used for study.

Pathogenicity.

Determination of the pathogenicity of the strains was outside the scope of the investigation. The effect of an early generation of the spinal fluid culture was tried on a rabbit and a guinea pig, and some additional information upon the relative pathogenicity of the recently isolated strains from the other sources was obtained from the immunization of rabbits. A rabbit, two-thirds grown, received an intravenous injection of a salt solution suspension of the entire growth of a 24 hour brown blood agar culture of the second generation; the inoculation was without effect. Instillation of a heavy suspension of the spinal fluid strain into the nose of a guinea pig was also without effect. Since the invasive spinal fluid strain had proved to be

[13] Avery, O. T., *J. Am. Med. Assn.*, 1918, lxx, 17.

non-pathogenic for the first rabbit used, suspensions of living cultures of the other strains were used for the immunization of rabbits. The rabbits injected with the strains isolated from the blood, throat, and nasopharynx died after one injection. In all three instances, *Bacillus pfeifferi* of typical morphology and cultural characteristics was isolated from the heart's blood.

Immunization.

For the production of immune serums, rabbits one-half to two-thirds grown were used. The two animals which had withstood the effect of the first intravenous injection of living suspensions of the spinal fluid and nose strains were subjected to further injections. Since the strains isolated from the blood, throat, and nasopharynx had proved fatal to rabbits injected intravenously with suspensions of living organisms, the immunization of other rabbits was begun with suspensions of these strains killed by heating to 80°C., living suspensions being used later. An additional rabbit was immunized with the spinal fluid strain. The intravenous injections were repeated every other day. When trial bleedings from the ear vein gave satisfactory agglutination, the animals were bled from the heart[14] and the serums were separated. In this way there were prepared two serums against the spinal fluid organism, and one serum against each of the other strains. These serums were used for a study of agglutination, complement fixation, and phagocytic activity.

Agglutination.

The macroscopic method was used. Each immune serum was used with its homologous strain and with the four heterologous strains. The serum was used in dilutions of 1:4, 1:8, 1:16, and so on by geometrical progression to 1:4,096. To each dilution of serum an equal quantity of *Bacillus pfeifferi* suspension was added, the total volume of fluid being 0.5 cc. This suspension was obtained by washing down twenty-four cultures with normal salt solution. The growths, since they were quite cohesive, were well shaken and then centrifuged for a few minutes to throw down the larger particles, and the supernatant fluid was used. A test was incubated at 56°C. for

[14] This was always done under anesthesia.

4 hours and placed in the ice box over night; another was incubated at 53°C. for 20 hours. Neither of these, however, proved so satisfactory as material incubated at 37°C. for 2 hours and placed in the ice box over night. Since it was feared that the strains might die out during subsequent work, cultures were made on brown blood agar slants in Blake flasks. From the luxuriant growths thus obtained large amounts of suspension were made, the organisms being killed by heating to 80°C. It was hoped that the use of such stock suspensions throughout all the work might give more satisfactory results. It was soon found, however, that agglutinations with these older suspensions were unsatisfactory. A slightly turbid fresh suspension of a 24 hour living culture gave better results than a more turbid suspension. The results of the agglutination tests are given in Table I,

TABLE I.

Agglutination.

Suspensions of *B. pfeifferi.*	Immune serums.						Normal rabbit serum.
	Spinal Fluid 1.	Spinal Fluid 2.	Blood.	Nose.	Throat.	Naso-pharynx.	
Spinal fluid.....	1:4,096	1:1,024	1:64	1:64	1:64	1:16	0
Blood.....	1:8	1:32	1:128	1:4	1:4	1:4	0
Nose.....	1:64	1:128	1:32	1:64	1:32	1:8	0
Throat.....	0	0	0	0	1:1,024	0	0
Nasopharynx.....	1:64	1:64	1:16	1:256	1:8	1:512	0

the figures being the highest dilution of serum at which clumping was apparent to the naked eye.

The results of the agglutination experiments show a wide variation in the titer of the serums for their homologous strains. Whether this is due to differences in agglutinability of the strains, to variations in agglutinogen content of the strains, or to variations in the response of the animals used it is impossible to decide. With the exception of the serum against the strain from the nose each serum agglutinates best its homologous strain. Each serum except that against the throat strain gives also cross-agglutination with the heterologous strains in low dilutions of serum. The serums against the spinal fluid organism have the highest titer, that against the strain from the throat is most specific, whereas the serums against the strains from

the nose and nasopharynx are least specific. It was originally intended to perform absorption agglutination experiments, but the exhaustion of the supply of serums and the later loss of some of the strains prevented this.

Complement Fixation.

In the complement fixation experiments two series of tests were set up. In the first, each immune serum was used with the spinal fluid strain. This exhausted the supply of serums, so that cross-fixation tests with the remaining strains as antigens could not be done. There was left just enough immune serum from the rabbits injected with the nose and throat strains to set up these serums with their homologous strains. After the original supply of serums was exhausted, an attempt was made to obtain a fresh supply by reimmunizing the original animals with stock killed suspensions of the strains which had been used before. This second lot of serums was not so satisfactory as the first but was used in a second series of experiments in which each serum was set up with all the strains. Because of the loss of several of the strains it was necessary to use as antigens the stock killed suspensions which had been prepared at the beginning of the work.

Undiluted serums heated at 56°C. for 30 minutes were used. In the first series the antigens were made of unheated bacterial suspensions such as were described under Agglutination. The system used was the anti-sheep rabbit in one-tenth the volume of the original Wassermann test. The serum, antigen, and complement, which consisted of two units of fresh guinea pig serum, were incubated at 37°C. for 1 hour. Two units of previously titrated anti-sheep amboceptor and a 5 per cent suspension of sheep corpuscles were then added and the whole was incubated for 1 hour. Antigen, serum, and hemolytic control tests were made each time. Serum and complement were kept constant; the antigens were varied, the figures given in Tables II and III being the fractions of the anticomplementary unit which gave complete inhibition of hemolysis.

As has been found to be the case in comparative studies of agglutination and complement fixation with other species of bacteria, the complement fixation experiments with *Bacillus pfeifferi* show a lesser

degree of specificity than do the agglutination reactions. An unexpected result is the degree of fixation which normal rabbit serum gave with the different antigens. The serum of a number of different normal rabbits was used, but always with the same result. In the second series, in which the older stock suspensions were used as antigens, this phenomenon was even more marked. It would appear to be due to a property of the antigen rather than of the normal serum;

TABLE II.

Complement Fixation.

Antigens of *B. pfeifferi.*	Immune serums.						Normal rabbit serum.
	Spinal Fluid 1.	Spinal Fluid 2.	Blood.	Nose.	Throat.	Naso-pharynx.	
Spinal fluid.....	$\frac{1}{256}$	$\frac{1}{256}$	$\frac{1}{4}$	$\frac{1}{64}$	$\frac{1}{128}$	$\frac{1}{64}$	$\frac{1}{4}$
Nose.....				$\frac{1}{64}$			$\frac{1}{4}$
Throat.....					$\frac{1}{64}$		$\frac{1}{4}$

TABLE III.

Complement Fixation.

Antigens of *B. pfeifferi.*	Immune serums.						Normal rabbit serum.
	Spinal Fluid 1.	Spinal Fluid 2.	Blood.	Nose.	Throat.	Naso-pharynx.	
Spinal fluid.....	$\frac{1}{32}$	$\frac{1}{128}$	$\frac{1}{8}$	$\frac{1}{16}$	$\frac{1}{32}$	$\frac{1}{16}$	$\frac{1}{8}$
Blood.....	$\frac{1}{8}$	$\frac{1}{8}$	$\frac{1}{8}$	$\frac{1}{8}$	$\frac{1}{8}$	$\frac{1}{8}$	$\frac{1}{8}$
Nose.....	$\frac{1}{32}$	$\frac{1}{32}$	$\frac{1}{16}$	$\frac{1}{64}$	$\frac{1}{64}$	$\frac{1}{16}$	$\frac{1}{8}$
Throat.....	$\frac{1}{32}$	$\frac{1}{32}$	$\frac{1}{8}$	$\frac{1}{32}$	$\frac{1}{32}$	$\frac{1}{32}$	$\frac{1}{8}$
Nasopharynx.....	$\frac{1}{32}$	$\frac{1}{32}$	$\frac{1}{32}$	$\frac{1}{16}$	$\frac{1}{16}$	$\frac{1}{32}$	$\frac{1}{16}$

the antigen appeared to be somewhat anticomplementary when used with normal rabbit serum, although it had no such action when added to the hemolytic system in the absence of rabbit serum.

Opsonins.

The opsonic index of each immune serum with its homologous influenzal strain was determined. Equal parts of suspensions of living *Bacillus pfeifferi*, normal rabbit leucocytes (collected in 0.2 per cent sodium citrate, washed, and suspended in normal saline

solution), and inactivated serum were mixed and incubated at 37°C. for 30 minutes. 50 polymorphonuclear leucocytes were counted and the number of cells taking part in phagocytosis was noted and compared with a similar series in which normal rabbit serum replaced the immune serum. The strain isolated from the blood was also set up with all the immune serums. The results of these experiments are given in Table IV. Because of the low opsonic indexes obtained in these experiments further work in this direction was not done.

With the exception of the first spinal fluid serum, all the serums used were of the second lot. The opsonin experiments show much the same kind and degree of variation as the agglutination tests. The spinal fluid serums, which had the highest agglutinin titers, have

TABLE IV.

Opsonins.

Suspensions of *B. pfeifferi.*	Immune serums.					
	Spinal Fluid 1.	Spinal Fluid 2.	Blood.	Nose.	Throat.	Naso-pharynx.
Spinal fluid	2.1	1.9				
Blood			1.4			
Nose				1.8		
Throat					1.5	
Nasopharynx						1.6
Blood	1.3	1.2	1.4	1.1	1.0	1.0

also the greatest opsonic activity. In the series in which the different serums were used with the same bacterial suspension, the organism being the strain from the blood, the action of the serum is best with the homologous organism, but the differences are not very marked.

DISCUSSION.

Immunologic studies of *B. pfeifferi* have led to conflicting results. Park,[15] by agglutination methods, found that cultures from different cases, even in the same locality, differed essentially or completely from each other. Isolations from the same case were usually identical, but different strains could occasionally be obtained from the same case. The differences noted were stable and persisted after

[15] Park, W. H., *J. Am. Med. Assn.*, 1919, lxxiii, 318.

subculturing. Huntoon and Hannum[16] came to exactly opposite conclusions and claimed an intimate relation for different strains, even when these were isolated in widely separated regions. Their work was based upon a small number of strains. Their agglutinin titers with three monovalent serums and four strains were low, the maximum of 1:640 being reached only once. The absorption experiments gave a decrease in titer varying from 1:10 to 1:80. Small and Dickson[17] also found strain relations by agglutination methods and arranged their strains into four groups. The number of strains used by them was, however, only ten, of which three fell into one group, four into another, while the two remaining groups were each represented by only one strain; there was one additional strain which could not be placed in any of these groups because of its inagglutinability. The propriety of setting up groups with only single representatives must appear doubtful; we believe that the study of a larger number of strains might have increased the number of single strain groups indefinitely. Valentine and Cooper,[18] in agglutination and absorption experiments on 171 strains from autopsies and from the upper air passages of patients with influenza, found little or no evidence of identity and concluded that under *B. pfeifferi* is included a heterogeneous group of organisms, among which there may be small subgroups. Six strains isolated from as many members of a single family, all taken ill with influenza at about the same time, were all different. Utheim[19] isolated thirty strains from thirty patients and tested the agglutination of the patients' serums with the homologous strains. Eleven (36 per cent) gave a positive agglutination in dilutions which varied from 1:20 to 1:160. In cross-agglutination experiments only one strain was agglutinated by heterologous serum, giving a positive result with the serums of three patients in 1:40 dilution. Utheim concluded that "each strain seemed to be individual in its immunologic reaction." Bell[20] studied the agglutination reactions of thirty-six strains, isolated by pharyngeal culture, with twenty-seven monovalent serums. His results are particularly valuable because of the high titers which the serums developed. Cross-agglutination occurred frequently in low dilution, but in only a few instances in higher dilutions. Absorption experiments also showed such variation that grouping of strains was impossible; the organisms used for absorption might absorb all, part, or none of the agglutinin for other strains. Strains isolated from two brothers were identical; from one individual two strains were isolated which were identical with two isolated from another individual. Three strains isolated from a single individual were quite distinct from one another. Although identical strains may occur, Bell concluded that "the influenza bacillus represents a heterogeneous group of

[16] Huntoon, F. M., and Hannum, S., *J. Immunol.*, 1919, iv, 167.
[17] Small, J. C., and Dickson, G. K., *J. Infect. Dis.*, 1920, xxvi, 230.
[18] Valentine, E., and Cooper, G. M., *J. Immunol.*, 1919, iv, 359.
[19] Utheim, K., *J. Infect. Dis.*, 1920, xxvii, 460.
[20] Bell, H. H., *J. Infect. Dis.*, 1920, xxvii, 464.

organisms." Cooke[21] was likewise unable to find any antigenic relation when sixteen of the strains isolated by Bell were used as antigens in the complement fixation reaction with patients' serums.

The study of five strains isolated from the same case, of which strains two were definitely invasive, appeared to offer better possibilities of obtaining knowledge of the biology of *Bacillus pfeifferi* than the study of many strains from a large number of cases. Whether the primary invasion in the present case was one of the blood stream or of the meninges, it is impossible to say. We believe it more probable that the meninges were invaded first and directly from the upper air passages, and that the blood was invaded from the meninges. The five strains isolated show distinct immunologic differences. The strains isolated from the nose and the nasopharynx show a greater apparent relation than any other two strains, and there may be some slight relation between the spinal fluid and the nose strains. The relation in each instance, however, is less marked than that which occurs among certain members of the colon-typhoid intermediates which are considered to be distinct species. Unfortunately, absorption experiments could not be done, but it is believed that they would not have added much of importance since cross-agglutination was so slight. The opsonin studies, although phagocytosis was not strikingly increased by the immune serums, show much the same differences as those obtained by agglutination. Since the results given were obtained by comparing the action of immune serum on normal leucocytes with that of normal serum, the phenomenon of spontaneous phagocytosis in the absence of serum, noted by Davis,[22] may be excluded. The pathogenicity of the five strains for rabbits also varied, although no great importance may be attached to these results because of the small number of animals used. Suspensions of living cultures of the blood, throat, and nasopharynx strains caused death from bacteremia. The nose and the spinal fluid strains were non-pathogenic for rabbits in large doses.

Our results, as well as those of Park,[15] Valentine and Cooper,[18] Utheim,[19] Bell,[20] and Cooke,[21] are directly opposed to those of Hun-

[21] Cooke, J. V., *J. Infect. Dis.*, 1920, xxvii, 476.
[22] Davis, D. J., *J. Am. Med. Assn.*, 1907, xlviii, 1563.

toon and Hannum,[16] and of Small and Dickson.[17] The evidence obtained from strains isolated from the same case or from different cases against identity of strains, and even against any close immunologic relation of strains, appears overwhelming. What bearing these results have upon the question of the etiological relation of *Bacillus pfeifferi* to the epidemic disease, it is difficult to say. Park[15] maintains that epidemic strains must be biologically identical if they are to be considered to have any causal connection with the disease. For most species of bacteria this is undoubtedly true. With *Bacillus pfeifferi* the question arises whether it may be possible that the wide variations in strains indicate biological instability which, under proper conditions, becomes an important factor in the causation of epidemics. In spite of the fact that immunologic characters become fixed upon cultivation of *Bacillus pfeifferi* outside the body, it is difficult to exclude the possibility that the organism is so labile that it may undergo changes under natural conditions in the human body. Although different strains, even from the same case, may show wide variations in experimental immunologic reactions, we believe that such strains are not so distinct from each other as are the various members of the colon-typhoid intermediate group, for instance. While the present case yielded five strains which have little in common except cultural and morphologic characters, it is probable that the subculturing of more colonies from the original cultures would have yielded a still larger number of immunologically different strains. However great the differences between the strains which were isolated, it is difficult to believe that these strains are not genetically the same.

If, as appears to be the case with antimeningococcus serum, the agglutinin and opsonin titers of an immune serum are a measure of the therapeutic value of the serum, the multiplicity of strains or races of *Bacillus pfeifferi* would seem to render impossible the preparation of a serum which might be of value in influenzal meningitis. Such a serum was prepared by Wollstein[23] at The Rockefeller Institute and was found to have definite value in experimental influenzal meningitis of monkeys. The serum had a low agglutinin titer but

[23] Wollstein, M., *J. Exp. Med.*, 1911, xiv, 73.

markedly increased phagocytosis. The immunologic relation of the organisms used in the experimental infection to those used in the preparation of the immune serum is not apparent from her report. The use of this serum in one of the cases reported by Torrey[4] was followed by recovery. Packard's[10] case also received this serum and recovered. Hills'[12] patient who did not receive the serum until the 4th day of the disease died on this day. Although one hesitates to draw conclusions from such a small number of cases, recovery may have been due to the serum, since the usual mortality of influenzal meningitis is approximately 90 per cent. In view of later immunologic studies, there is no reason to suppose that the organisms from the recovered cases would have been found to be identical with the strains used in the preparation of the serum. The present opsonin experiments with monovalent serums indicate strain variations comparable with those obtained in the agglutination experiments. The opsonin titers of our serums were so low that we do not attach great importance to the results obtained; it is possible that serums with higher opsonin values might have shown less variation between strains and a greater degree of group reaction. It is also possible, if strains of *Bacillus pfeifferi* are genetically related but immunologically distinct, that the therapeutic value of a serum in meningitis may depend upon properties other than those which can be measured by agglutination or opsonin reactions.

SUMMARY.

Five strains of *Bacillus pfeifferi* were isolated from a case of meningitis. These strains came from the spinal fluid, blood, nose, throat, and nasopharynx.

Immunologic reactions show no definite relations between these strains, although those from the nose, throat, and nasopharynx might be presumed to be related to one another. It is also presumable that the spinal fluid strain was derived from the upper air passages, and that the blood was invaded from the meninges. In spite of immunologic differences, it is believed that the five strains were genetically related.

The variations in these five strains from a single case are as great as those which have been found by others for strains from different cases, or individuals.

As determined by immunologic reactions, the number of so called strains of *Bacillus pfeifferi* is apparently limited only by the number of cultures which have been or might be isolated. It is inconceivable that under the designation *Bacillus pfeifferi* is included a heterogeneous mixture of innumerable distinct races of bacteria; there must be some biological relation which cannot be established by agglutination, complement fixation, or opsonin reactions.

The variations which have been noted may be an indication of a degree of instability which may have some bearing upon questions relating to the epidemiology of *Bacillus pfeifferi* infections and to the serum therapy of influenzal meningitis.

The strain variations which have been shown to exist by immunologic methods do not support the theory of the etiological relation of *Bacillus pfeifferi* to epidemic influenza, unless it can be shown that such variations are due to instability of the organism. The fact that the incidence of meningeal involvement was only slightly increased during the last pandemic is also evidence against the causal relation of the organism to the pandemic disease.

THE INCIDENCE OF BLACKHEAD AND OCCURRENCE OF HETERAKIS PAPILLOSA IN A FLOCK OF ARTIFICIALLY REARED TURKEYS.

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During the season of 1920 a flock of turkeys reared in incubator and brooder, and on new soil, has provided further data on the incidence of blackhead under such circumstances and has furnished additional evidence that this method is a successful means of rearing turkeys. Interesting data have also been obtained on the occurrence of the worm (*Heterakis papillosa*) shown in previous work to be a factor in the production of this disease and some evidence has been obtained on the source of this worm in the environment under which the experiments were conducted.

The ground on which the flock was reared was in a large, well fenced horse paddock which had not been used for turkeys or chickens for a period of more than 3 years. The soil was plowed and sown in the spring. The old turkeys and poultry were confined in enclosures at a distance from the paddock and precautions were taken to prevent attendants carrying infection from these to the flock in the paddock. As in the previous field experiments no attempt was made to exclude wild birds.

Three lots of eggs were incubated in commercial incubators. These yielded 85 poults. The lots were brooded separately and kept apart in coops and enclosures of their own until August 9 when they were combined into one flock and given the run of the paddock, within which the enclosures used up to this time had been located. Lot 1 was hatched May 30, Lot 2 June 9, and Lot 3 June 22. They were placed outdoors June 9 and 15, and July 1 respectively.

Up to October 1, nine had died of miscellaneous causes and four from blackhead. Twenty-eight in all had been removed at intervals

for experimental purposes. The remaining birds were in a flourishing condition. Of those that died of miscellaneous causes, one was trampled to death, in two the cause of death remained undetermined, two died of general weakness, two of leg weakness, one of typhlitis, and one of enteritis. After October 1, one other case of blackhead occurred and one more bird was removed for experimental purposes.

It will be noted that, in all, five cases of blackhead occurred in the flock. The dates on which the birds became sick were July 14, September 3, 15, 17, and October 5. The contents of one cecum in the first case were washed and sedimented for worms and one young *Heterakis papillosa* was found. The second case was not examined for worms. The contents of both ceca of the three remaining cases were washed and sedimented, and ten *Heterakis* small to full grown were found in one, one young and one full grown in another, and two full grown specimens in the third. Three of the birds were examined for coccidia with negative results.

With the exception of four birds retained for breeding, the remainder of the flock consisting of 42 birds was killed for food purposes during November and December. Data on these turkeys are given in Table I. The organs of all were examined for lesions of blackhead and for scars. The examination proved entirely negative. None of these birds had at any time shown symptoms of illness. All were normal at autopsy with one exception, in which the mucosa at the tips of the ceca was congested and pigmented.

The degree of infestation with *Heterakis* was determined by washing and sedimenting the cecal contents. It will be noted from the table that five were free of worms. Eleven turkeys harbored one worm each; eight, two worms; three, three worms; six, four worms; one, seven worms; three, eleven worms; and one, fourteen worms. As evidence of the difficulty the parasite has in establishing itself in a flock on virgin soil, it is of interest to note the number of cases in which either males alone or females alone were present. In twelve birds only males were present and in six only females. Further obstacles are presented to the multiplication of the parasite in that males alone presumably might occur in one cecum and females in the other, and moreover when both sexes occur together, individuals of both in the same stage of development might not be present, a circumstance not favorable to reproduction.

TABLE I.

Data on Turkeys Killed for Food.

No. of turkey.	Date killed.	Live weight.	Color.* Sex.*	No. of *H. papillosa* present.
	1920	*lbs.*		
430	Nov. 4	9½	B.	1 grown, 1 almost grown.
431	" 20		" M.	2 " females.
432	" 20	13¾	" "	None.
433	" 20	9½	W.; F.	"
434	" 20	8¼	B.; "	"
435	" 22	13¾	W.; M.	2 adult males.
436	" 22	13	" "	2 " females, 1 male.
437	" 22	12	" "	4 " " 6 nearly to full grown, 1 young male.
438	" 22	8	" F.	1 adult female, 3 young to grown males.
439	" 23	16½	B.; M.	1 young male.
440	" 23	13½	" "	3 grown females, 4 adult males.
441	" 23	14	" "	3 " males, 1 young female.
442	Dec. 18	13	" "	1 male nearly grown.
443	" 18	13½	" "	5 males and 5 females nearly to full grown, and a young stage 2–3 mm. long.
444	" 18	8¼	W.; F.	1 grown male.
445	" 18	8¼	B.; "	4 adult males.
446	" 18	9¼	" "	1 male and 1 female, adults.
447	" 20	15	W.; M.	1 grown male.
448	" 20	8¾	B.; F.	3 " females and 8 males nearly to full grown.
449	" 20	14¼	" M.	1 adult female.
450	" 20	14	" "	1 grown male.
451	" 20	16¼	W.; "	3 adult females and 1 male nearly grown.
426	" 21	9	B.; F.	2 grown females.
452	" 21	9¾	W.; "	1 " female, 1 adult male.
453	" 21	8	B.; "	14 males and females, nearly to full grown.
454	" 21	9½	" "	2 grown males.
455	" 22	10½	" "	1 nearly grown female.
456	" 22	10½	" "	1 " " male.
457	" 22	8½	" "	3 females nearly to full grown.
428	" 22	8½	" "	3 adult females, 1 male nearly grown.
458	" 22	10½	" "	1 grown female, 2 adult males.
459	" 23	12	W.; M.	1 " male.
460†	" 23	14¼	B.; "	None.
461	" 23	9¾	" F.	4 worms.
462	" 28	14	" M.	1 nearly grown male.
463	" 28	16	" "	2 " " males.
464	" 28	16¼	" "	None.
465	" 28	16½	" "	1 nearly grown female.

* B. indicates bronze; W., white; M., male; F., female.

† Mucosa of tips of ceca greatly congested and pigmented.

As evidence that the flock of turkeys was not one specially resistant to blackhead it will be of interest to consider the history of a number withdrawn during June and July and exposed to an old flock. The data on the incidence of blackhead and the occurrence of *Heterakis* in these furnish a striking contrast to what was observed in those not exposed.

Of twenty-nine birds removed for experimental purposes, sixteen were penned with the old flock beginning in July. All became

TABLE II.

Data on Turkeys Exposed to Old Flock.

No. of turkey.	Date exposed.	Date sick.	Blackhead.	Cecal contents washed and sedimented.	No. of *H. papillosa* present.
	1920	*1920*			
400	July 12	Aug. 2	+	Both ceca.	59 young to grown.
401	" 12	" 16	+	" "	21 "
402	" 12	" 8	+	" "	7 " to grown.
403	" 12	July 26	+	" "	About 25 young.
404	" 12	Aug. 16	*		
405	" 12	July 29	+	Both ceca.	11 young.
406	" 12	Aug. 8	+	" "	30 "
407	" 12	" 2	+	" "	90 "
408	" 19	" 6	+	†	
409	" 19	" 8	+	Both ceca.	100 young.
410	" 19	" 23	+	†	
411	" 19	" 16	*		
412	" 12	July 28	+	Both ceca.	7 young.
413	" 12	Aug. 8	+	" "	7 nearly grown.
414	" 12	" 23	+	" "	40 young to grown.
415	" 12	" 16	+	One cecum.	Many, mostly young.

* Survived.

† No examination for worms.

sick within 42 days (Table II). Two survived and there is every reason to believe that they underwent an attack of blackhead, since both were sick for about 7 days. The diagnosis of blackhead in the others was confirmed at autopsy. In washing and sedimenting the contents of the ceca the number of *Heterakis* present was determined. In eight birds the number of worms ranged from 21 to 100. In the four remaining ones examined, the number found was 7 to 11. It should be stated that the method used in collecting the worms

would not lead to the detection of very young stages. Ten of the birds were examined at autopsy for coccidia with negative results.

Since the flock of normal turkeys was protected from infestation with *Heterakis papillosa* from other turkeys and chickens, the source of this parasite becomes a question for consideration. We have been fortunate in locating at least one of the sources of this infection in the ring-necked pheasant, present in this locality. In the previous fall a pheasant killed on a neighboring farm harbored specimens of this worm. Pheasants have been observed from time to time on the Institute farm and on a number of occasions they have been seen in close proximity to the laboratory and in the paddock in which this year's flock was reared. In June of this season attendants reported on a number of occasions a pair of pheasants present in this paddock. On November 18, a pheasant that had fallen a victim to hunters was found dead in the paddock and at autopsy specimens of *Heterakis papillosa* were found in the ceca. Considering the resistant character of the ova of this parasite, there seems little question that this bird, although present in small numbers, is capable of maintaining a certain degree of soil infestation with this parasite. Fortunately, this does not appear to be sufficiently concentrated to interfere with the successful rearing of turkeys when the soil has been subjected to ploughing and cultivation incident to planting. It will, however, be a safe practice to discourage the visits of this bird.

Experiment on the Persistence of the Ova of Heterakis in the Soil.

An enclosure that had proved infectious to turkeys during the season of 1919[1] remained occupied by recovered cases until January 21, 1920, at which time it became vacant and remained closed until June 28, a period of a little over 5 months. This covered a time of exceptionally severe winter weather with much ice and snow. On the latter date, four healthy turkeys, 29 days old, from the normal flock referred to above were placed in the enclosure. These turkeys contracted blackhead in 11, 21, 23, and 28 days respectively. *Heterakis* was present in all. The contents of both ceca were washed and

[1] Smith, T., and Graybill, H. W., *J. Exp. Med.*, 1920, xxxi, 633, Experiment 12, *b*.

sedimented in the case of three of the birds. In one, seven worms were collected, and in the remaining two, many were found. Only one cecum of the fourth bird was examined and twenty-seven young *Heterakis* were collected.

Although the enclosure during the time it was unoccupied remained accessible to wild birds on the wing, the conditions for visits of hosts of *Heterakis* were on the whole unfavorable. Circumstantial evidence that the ova in the soil had survived and had not been introduced during the interval is furnished by the fact that the number of worms found per bird corresponded with that of the poults penned with the old flock and not with that of the flock on new ground, which was much more favorably located for the visits of birds.

SUMMARY.

In a flock of artificially reared turkeys originally consisting of 85 birds and reduced during the summer and fall by deaths and withdrawals for experimental purposes to 42 birds, five cases of blackhead occurred. These appeared during the months of July, September, and October. In four, *Heterakis* was searched for and found. In 38 birds from this flock killed for food during November and December, five harbored no *Heterakis*, and the rest carried light infestations.

Of sixteen healthy birds withdrawn from the above flock during July and placed with a flock of older birds which had passed through this disease in former seasons, all contracted blackhead and fourteen died of the disease. The infestation with *Heterakis* was, as a rule, high, reaching a hundred specimens in some cases. In general, it appears that a high infestation with *Heterakis* is correlated with a high incidence of blackhead, a relation that had already been inferred in feeding experiments. In both of these groups no other species of worm was found in the ceca, and in instances in which examinations for coccidia were made none was found.

Pheasants have been incriminated as a source of infestation with *Heterakis papillosa* in artificially reared flocks.

In an artificially reared flock 38 birds that had never been ill, when killed in November and December, failed to show lesions of blackhead or evidence in the nature of scars that they had passed through an attack of the disease.

Infectious soil that had remained unoccupied by turkeys and chickens for a period of 5 months beginning in the depth of a severe winter still harbored viable ova of *Heterakis* and proved highly dangerous to young poults.

These experiments and observations fail to throw any light on the source of the protozoan parasite (*Amœba meleagridis*) which causes the fatal lesions of blackhead.